Thesis for doctoral degree (Ph.D.)
2015

Strengthening the Pillars of Cancer Immunotherapy

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The cover was painted by artist George Tapu (el_tzap @ fiverr.com) and depicts scaffolding set up against the pillars of a Roman era temple.

Published by Karolinska Institutet.
Printed by Eprint AB 2015

ISBN 978-91-7676-015-4

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THESIS FOR DOCTORAL DEGREE (Ph.D.)

Cancer Center Karolinska (CCK) Lecture Hall, R8:00,

Karolinska University Hospital,

Stockholm

Friday the 28th of August, 2015 at 09:00

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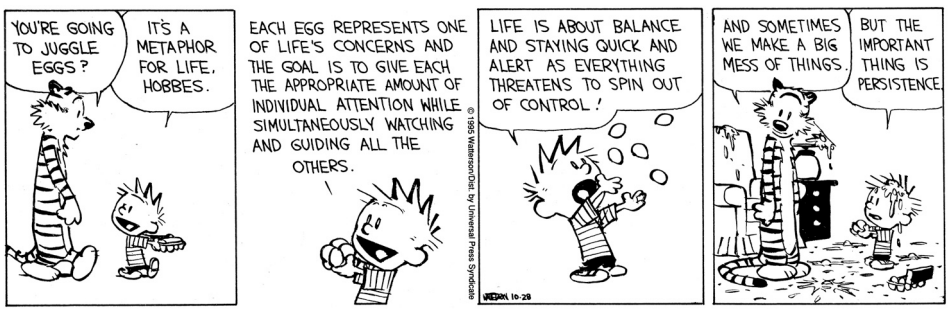
Voor Mindy

Just one flight, happiness



FOREWORD

Six years ago I came to Sweden on an adventure to learn tumor immunology under the tutelage of Prof. Rolf Kiessling. When I started, I came to the lab knowing nearly next to nothing of the rudiments of immunology. After a couple of years I felt my grasp on this field was tightening, but only recently have I come to the understanding that knowing tumor immunology is not enough. The work I have done, as is partially represented in this thesis, requires the foundations that have been laid by ingenious immunologists, transplantation and tumor biologists. For example, Macfarlane Burnet and his seminal "The clonal selection theory of acquired immunity". But beyond building on "correct" work, we are building on the rubble of many unsuccessful but required endeavors. The course of any PhD students' tribulation is littered with the rubble of many failed experiments. And yet, it is these failed experiments that are the guide toward success. Ask Rolf how many times we had to sit down and discuss why his favorite ^{51}Cr cytotoxicity assay did not work in my hands. One must also not forget that rubble plays an important role in the construction of magnificent buildings as well. The history of tumor immunology and immunotherapy is rife with failure and tons of rubble. Nonetheless, tumor immunologists have not lost faith in our collective work, and many have built fantastic foundations for future work. I have recently come to the realization of the importance of these historical works, both those that are foundational as well as rubble. In this thesis I hope to shed some light on the researchers that laid the solid foundations that we build on.

One thing that my colleagues will be happy to share with you has been my enthusiasm for asking questions. To be honest, it may very well be that I am too insistent with my enduring inquires (my apologies dear colleagues), nonetheless I believe that this is an essential part of being who I hope to become, a competent researcher. Often it has been the case that someone has given a fantastic presentation that does not spawn any discussion or questions. This is painful to see, particularly when a group of researchers are present, even more so when it is a group of PhD students, but also when laypeople are present. Our research is one that has direct application for the lives of many people. I wonder if it is embarrassment of lacking knowledge that precipitates a lack of participation. To this end, I have added to this thesis an extended layman's introduction that I hope will allow for the participation of a greater audience in the discussion of cancer and cancer therapy.

ABSTRACT

The treatment of disease in cancer patients by harnessing the potent mechanisms that exist within the immune system is not a novel approach, and has been first attempted more than a century ago. During the major part of this century cancer immunotherapy has been relegated to the periphery of standard care for patients. Within the last few years a dramatic shift has occurred in the treatment of cancer, and patients are now the recipients of drugs and therapies that aim to modulate and modify their immunity towards cancer. These treatments fall into one of two categories; either the therapy is passive immunotherapy or active immunotherapy. The first is based on the premise of introducing specific immunity in the form of cytokines, monoclonal antibodies or tumor specific T cells into the tumor-bearing patient. Active immunotherapy aims at inducing an *in vivo* tumor specific response, typically through various means of vaccination to activate specific immunity. We have directed our efforts at strengthening the pillar of passive immunotherapy through harnessing our understanding of the tumor microenvironment. Tumors generate large amounts of reactive oxygen species which adversely effect anti-tumor effector T cells. One approach to mitigate the effects of reactive oxygen intermediates is by co-expressing high levels of catalase in tumor-redirected T cells that express chimeric antigen receptors. Increased levels of catalase neutralize the negative effects of oxidative stress on T cells and allows them to survive, proliferative and perform their cytolytic functions whereas typically they would become anergic. In addition, these cells are able to protect bystander T and NK cells from oxidative stress mediated dysfunction. This strategy of attenuating the negative effects derived from the tumor microenvironment can potentially increase the efficacy of chimeric antigen receptor based passive immunotherapy. To strengthen the pillar of active immunotherapy we attempted to identify, enhance and broaden the potential targets of DNA based vaccine delivery. Vaccines, that activate immunity against tumor antigens, have the potential to revolutionize the field of cancer treatment. DNA vaccines in particular remain an interesting platform for activating tumor specific immunity. The delivery of DNA vaccines into the skin, where professional antigen presenting cells that can be readily primed are present, can induce recruitment of tumor specific T cells as well as antibody producing B cells. We found that dermal DNA vaccination relies heavily on NF-κB activation but surprisingly not on the IRF. IRF induces the production of type I interferon which are strong activators of antiviral activity in immune cells. This is particularly relevant for inducing anti-tumor responses, which are mediated in large part by cytotoxic T lymphocytes. To harness this mechanism we delivered a genetically encoded intracellular DNA sensing molecule, DAI, which increased type I interferon molecule production as well as matured skin resident dendritic cells. This led to increased anti-tumor T cell activity as well as provided long-term protection from tumor re-challenge by generating more abundant tumor specific memory T cells. Generation of vaccine responses against cancer requires targeting antigens expressed by the tumor. To increase the potential targets available to cancer immunotherapists we explored the capacity of eliciting an immunological response against oncofetal tumor antigen Cripto-1. Delivery of DNA vaccine encoding full length Cripto-1 into the dermis of mice generated a cellular as well as humoral response that was able to inhibit the growth of transplanted tumors as well as decreased metastatic burden.

The pillars of cancer immunotherapy rest upon foundations laid by a myriad of immunologists and cancer biologists. By protecting adoptively transferred tumor specific lymphocytes and furthering the understanding, as well as boosting the immunogenicity of classical and novel tumor antigen encoding DNA vaccines, we hope to improve the outcomes of cancer immunotherapy.

LIST OF INCLUDED STUDIES

I. **LIGTENBERG MA**, Mougiakakos D, Mukhopadhyay M, Witt K, Lladser A, Chmielewski M, Riet T, Abken H, Kiessling R. Co-expressed catalase protects chimeric antigen receptor-redirected T cells as well as bystander cells from oxidative stress-induced loss of anti-tumor activity. *Manuscript*

II. **LIGTENBERG MA**, Rojas-Colonelli N, Kiessling R*, Lladser A*. NF-κB activation during intradermal DNA vaccination is essential for eliciting tumor protective antigen-specific CTL responses. *Human Vaccines & Immunotherapeutics, 2013, 9:10, 2189-2195*

III. Lladser A, Mougiakakos D, Tufvesson H, **LIGTENBERG MA**, Quest AFG, Kiessling R* and Ljungberg K*. DAI (DLM-1/ZBP1) as a Genetic Adjuvant for DNA Vaccines That Promotes Effective Antitumor CTL Immunity. *Molecular Therapy, 2011, 19:3, 594-601*

IV. **LIGTENBERG MA**, Witt K, Conti L, Lanzardo S, Tufvesson-Stiller H, Ostling J, Sette A, Rolny C, Lladser A, Cavallo F, Kiessling R. Vaccination against tumor-associated antigen Cripto-1 elicits a protective immune response to metastatic melanoma and breast cancer stem cells. *Manuscript*

LIST OF ASSOCIATED STUDIES

I. **LIGTENBERG MA**, Çinar Ö, Holmdahl R. Mougiakakos D, Kiessling R. Methylcholanthrene-Induced Sarcomas Develop Independently from NOX2-Derived ROS. *PLoS One. 2015 Jun 15;10(6):e0129786*

II. Herrada AA, Rojas-Colonelli N, González-Figueroa P, Roco J, Oyarce C, **LIGTENBERG MA**, Lladser A. Harnessing DNA-induced immune responses for improving cancer vaccines. *Human Vaccines & Immunotherapeutics, 2012, 1:8(11), 1682-1693*

CONTENTS

LIST OF ABBREVIATIONS

ACT	Adoptive cell transfer
ADCC	Antibody-dependent cellular cytotoxicity
ADCP	Antibody-dependent cellular phagocytosis
Ago	Argonaute
AIDS	Acquired immune deficiency syndrome
AIM2	Absent in melanoma 2
ALL	Acute lymphoblastic leukemia
AP-1	Activator protein 1
APC	Antigen-presenting cell
ARG1	Arginase 1
BCG	Bacillus Calmette–Guérin
BCR	B cell receptor
CAIX	Carbonic Anhydrase IX
CAR	Chimeric antigen receptor
Cas	CRISPR-associated genes
CD	Cluster of differentiation
cDC	Classic DC
CDC	Complement-dependent cytotoxicity
cGAS	Cyclic GMP-AMP synthase
CLL	Chronic lymphoblastic leukemia
CLP	Common lymphoid progenitor
CML	Chronic Myelogenous Leukemia
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CR	Cripto-1
CRC	Colorectal cancer
CRISPR	Clustered regularly interspaced short palindromic repeat
CSC	Cancer stem cell
CSF	Colony stimulating factor
CSF-1R	Colony stimulating factor receptor one
CTL	Cytotoxic T lymphocyte
CTLA4	Cytotoxic T-lymphocyte-associated protein 4
CXCL8	IL-8
DAI	DNA-dependent activator of IFN- regulatory factors
DAMPs	Danger-associated molecular patterns
DC	Dendritic cell
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded Ribonucleic acid
ER	Endoplasmic reticulum
FasL	FAS ligand
FDA	US Food and Drug Administration
FoxP3	forkhead box P3
GM-CSF	Granulocyte-macrophage colony-stimulating factor
gp100	Glycoprotein 100
GVHD	Graft-versus-host disease
Gy	Gray
HER2/neu	Receptor tyrosine-protein kinase erbB-2

HMGB1	High-mobility group box 1
HPV	Human papillomavirus
HSC	Hematopoietic stem cell
HSCT	Hematopoietic stem cell transplantation
HSV	Herpes simplex virus
Id	Idiotype
IFI16	Interferon-gamma induced protein IFI 16
IFN	Interferon
Ig	Immunoglobulins
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF	Interferon regulatory factors
ITAM	Immunoreceptor tyrosine-based activation motif
IVT	In vitro transcribed
KIR	Killer-cell immunoglobulin-like receptor
KLH	Keyhole limpet hemocyanin
KO	Knock-out
LAG3	Lymphocyte-activation gene 3
LAK	Lymphokine-activated killer cell
LL-37	Cathelicidin
LPS	Lipopolysaccharide
LRRFIP	Leucine Rich Repeat In FLII Interacting Protein 1
MAGE	Melanoma antigen gene
MAM-A	Mammaglobin-A
MART1	Melanoma antigen recognized by T cells 1
MCA	Methylcholanthrene
mCR	Mouse CR
MDSC	Myeloid-derived Suppressor Cell
MHC	Major histocompatibility complex
MUC1	Mucin 1
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NF-κB	Nuclear factor kappa-light-chain-enhancer of B cells
NFAT	Nuclear factor of activated T-cells
NIH	National Institutes of Health
NK	Natural killer cells
NO	Nitric oxide
OVA	Ovalbumin
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PD-1	Programmed cell death protein 1
pDAI	DAI encoding plasmid DNA
pDC	Plasmacytoid dendritic cell
PD-L1	Programmed death-ligand 1
PD-L2	Programmed death-ligand 2
PGE2	Prostaglandin E2
PRR	Pattern recognition receptor
RCC	Renal cell carcinoma
RISC	RNA-induced silencing complex

RNA	Ribonucleic acid
RNAi	RNA interference
ROS	Reactive oxygen species
ssRNA	Single-stranded RNA
TAA	Tumor-Associated Antigens
TAM	Tumor-associated macrophage
TAP	Transporter associated with antigen processing
TBK-1	TANK- binding kinase 1
TCR	T cell receptor
TGF-β	Transforming growth factor beta
TIL	Tumor-infiltrating lymphocytes
TIM3	T-cell immunoglobulin domain and mucin domain 3
TLR	Toll-like receptors
TNM	TNM Classification of Malignant Tumours
TRAIL	TNF-related apoptosis-inducing ligand
Treg	Regulatory T cell
TRP2	Tyrosinase-related protein 2
VEGF	Vascular endothelial growth factor
WT	Wild type
WT1	Wilms tumor 1
ZFN	Zinc finger nuclease

A GUIDE TO "STRENGTHENING THE PILLARS OF CANCER IMMUNOTHERAPY"

I would like to take you on a short trip through time to a fictional Roman border town around the year 200 AD. Romans are of course famous for building prosperous towns with many buildings requiring strong pillars; this is a town similar to many other Roman towns. There is a central governing body made up of wealthy aristocrats, whom are busy administering and planning the future of the town. It also has many important local craftsmen: the farmer providing the food and energy for the townsfolk; the carpenter and stonemasons building the strong structures that keep the buildings standing; the bankers storing wealth around the town for later use; the merchants transporting goods from one part of the town to the other. Each is specialized at his skill and each being essential to the functioning of the town; all typically working together with the goal of ensuring the well being of the town.

And as with any prospering town in the 2nd century, barbarians and raiders are watching like wolves from the edge of the forest salivating at the idea of pillaging the town. The town of course is not helpless. As with any interesting story, the barbarians add a bit of conflict making this a juicy tale. The Romans have, after centuries of dealing with barbarians, developed many interesting methods of keeping them at bay. The first line of defense is the wooden palisade. This wooden wall is able to deter most barbarians and raiders from entering the town. But all is not lost if or when they are able to break in; the Romans still have a couple of natural barriers to help. Throughout the town and at the town walls there are complex spike traps laid out ready to snap shut on specific types of barbarians. When the barbarians break through the walls the citizens of the town scream out for help, alerting the soldiers that the walls have been breached. Standing watch on the walls are the Roman guards and sentinels, the guards rush to the aid of the citizens and directly confront the barbarians that break through the wall. The sentinels from the walls come to join in the brawl but during the fight they pick up armor and weapons from the dead barbarians and run to the camps where the Roman legionaries are waiting. These sentinels play a crucial role in waking up and helping to train the legionaries identify the barbarians they need to destroy. There are three main types of legionaries; the Velites: soldiers that would cast long javelins at the enemy; the Equites: soldiers that formed the cavalry that would strike directly at the enemy; and finally the Legionary: heavy infantry soldiers that would support both the Velites and Equites. Some barbarians would be more susceptible to the javelins of the Velites and others to the swords of the Equites. The Legionary infantry always aided both responses against the barbarians. The response of the legionaries would always take a while as a training process would be required, and until they could act, the guards, traps and the walls had to keep the barbarians at bay. Once the battle was

won, a great deal of the legionaries would retire, but some would remain in service and wait in the camps telling tales of their victory against the enemy and if they were required for their experience in tackling a specific barbarian again, they would be roused and would retrain a new army to return to the battle field.

Unfortunately, our story does not end there. During this age of Roman wealth and prosperity it would sometimes occur that one of the obedient citizens, the stonemasons, the aristocrats, the merchants, and sometimes even the legionaries themselves would become greedy and corrupt. While their neighbors would protest against the corrupt Romans telling them to stop stealing and that there are already enough of them in the town, the offenders would ignore these signals and take it upon themselves to attempt to take as much of the prosperity for themselves as they could. There are of course guards patrolling the town, checking in on the citizens, sometimes even arresting and killing unruly ones. Sometimes the legionaries dealt directly as vigilantes with these offensive citizens. But as often is the case, the corrupt and greedy would have a couple of tricks up their sleeves to keep the guards off their back. These include: directly killing of the guards with a stealthy knife to the gut or by making the environment hostile by intentionally throwing their foul waste in front of their houses making patrolling impossible, or even by recruiting and corrupting the guards to aid and abet their crime. Eventually, the corrupt citizens spread around the town using up all the resources, resulting in the Romans failing in their function to carry out their assigned tasks. Eventually this corruption would lead to the demise of our Roman border-town.

To illuminate what we have achieved in this thesis, I ask you to return to our imaginary Roman border-town prior to its demise due to the greedy corrupt citizens. There are two methods that are being used to help our Roman towns: the first by giving the town guards and legionnaires directly the tools to handle the problem; the second is by educating the town legionnaires to identify the offending citizens.

The first method that has been used effectively to cull offending citizens in other towns has been by endowing legionnaires with laser vision goggles, allowing them to easily identify their targets. This has worked very well against corrupted Velites (legionaries with the javelins), but citizens that live in one location, such as corrupted milkmaids, have been unsuccessfully treated. In the first part of this thesis we attempt to solve the problem that the legionnaires encounter when they visit the hostile surroundings of offending citizens, namely, all that foul waste they throw on the street. We train the legionnaires to brave this environment and put them in a boot camp and give them gas masks to make them resistant to the noxious stench that surrounds the corrupt citizens. In addition, we have given them laser vision goggles so that they can better see which are the disobedient citizens to cull. We show that even in the presence of foul waste, our re-trained legionnaires are able to survive and kill the corrupt citizens. These newly trained legionnaires were even able to

share their own gas masks with other fellow guards, allowing them to also spring into action (Paper I).

The second method, while exceedingly effective against barbarians, has been more difficult to realize against roman citizens, even those that are corrupt and offensive. For more than a century immunologists have been educating the towns defenses by showing the weapons/armor of the barbarians to the sentinels on the walls of the town. This allows the sentinels to train the legionnaires as to the appearance of the barbarians, and offers profound protection. In the second part of this thesis we describe a method to retrain the sentinels to recognize the identifying features of the enemy from within- i.e. the offending corrupt citizens and their wicked ways they use to overcome the sentinels. The sentinels are able to teach and train the legionnaires in their new skills at identifying corrupt citizens. We attempted to understand how the sentinels recognize the plans that were introduced and which of their perceptions were important in getting them to present the tools and cloths in a manner that elicits the legionnaires to become educated to kill the corrupt Romans. We found that they use only a particular set of senses to recognize assistance (Paper II). Using this knowledge we introduced a method for the sentinels to improve their recognition that would facilitate eradicating the offending citizens. The improved recognition consequently led to an improved legionary response that was able to eliminate the corrupt Romans and keep the town from its demise (Paper III). Finally, we were also able to show that we could arouse the legionaries to the weapons of the corrupt Romans which they used to spread throughout the town. We were able to identify which part of the weapons the Equites recognized as well as showing that the Velites produced javelins specific for this weapon. This training was able to protect a couple of different Roman towns from two types of corrupt citizen (Paper IV).

The findings contained within this thesis will join the scaffolding that allows for the strengthening of the pillars of cancer immunotherapy.

Cancer and the therapy of cancer is a shockingly commonplace occurrence that many of my own family and friends have had to confront. The diagnosis of cancer can be overwhelming and confusing. I believe that a great part of the overwhelming nature is derived from the common understanding that cancer is a complex and not wholly understood and treatable disease. This extended layman's abstract, I hope, will allow for a broader understanding of cancer and the basis of cancer immunotherapy. Of course the description given above also highlights the work we have been doing, but I believe that within the context of this metaphor many therapies and diseases can be understandably described.

1 FOUNDATIONS OF IMMUNOLOGY AND CANCER IMMUNOLOGY

All organisms known to exist on our planet survive in an environment in which they are confronted by constant competition. Their competitors come in the form of similar species competing for identical niches for survival, but they also come in the form of opportunistic parasitic organisms that invade their hosts. These parasitic organisms vary from large multicellular helminthes, single cellular bacteria to viruses. When these organisms cause dysfunction and are able to disseminate to new hosts they can be classified as pathogens. Both types of competition at a macro and at a micro scale are drivers of Darwinism. Over millennia this has led to the development of very complex anti-pathogen mechanisms, termed immunity. Previously, it was thought that immunity was only the domain of large complex multicellular animals, but now it can be argued that mechanisms of immunity can be found in all forms of life. Immunity can be categorized into two general terms, namely innate and adaptive immunity though there is much flexibility and flux between both categories. The innate immunity consists of, but is not limited to, peptidoglycan or cell walls in bacteria, cross-linked cellulose fibers in plants or outer epithelial layers in mammals. These are natural boundaries and mechanisms to prevent invasion of the organisms by potential pathogens. Adaptive immunity is a response generated within the organism that is able to specifically neutralize a single type of pathogen; in bacteria the CRISPR/Cas complexes neutralizes foreign DNA, in plants RISC/ago complexes neutralize foreign RNA, in mammals the TCR/MHC complexes neutralize foreign protein. The more complex the organism is, the greater the requirement and availability of the immune arsenal to eliminate disease.

While in general immunology of organisms is of great interest, human immunology is of primary concern for us. The first section of the introductory part of this thesis will lay the foundations of immunology and how the immune system interacts with cancer. The second section will focus on the current pillars of cancer immunotherapy and how researchers are harnessing the immune system to treat cancer. Finally, in the third section, I will describe how our work has strengthened these pillars as well as provide speculation on the form of cancer immunotherapy in the future.

1.1 IMMUNOLOGY

The study of the human immune system is a complex and constantly developing field. When pathogens invade the human host, the anti-pathogen response is mounted immediately; this response is broad in scope and potent enough to eliminate a variety of pathogens. Protection is mediated by direct as well as indirect cellular responses. These important players in immunity are leukocytes. Hematological cells have the highest turnover of all human cells. This requires the replenishment of these populations on a constant basis. To fulfill this requirement we have pluripotent hematopoietic stem cells (HSC), that in addition to maintaining their own self-renewal give rise to common lymphoid progenitors (CLP) and common myeloid progenitors (CMP). The CLP cells differentiate into natural killer (NK), plasmacytoid DC (pDC), B and T cells. The CMP cells can differentiate into either megakaryocyte/erythroid progenitors, which are responsible for red blood cell and platelet production, or granulocyte/macrophage progenitor that lead to the generation of mast, eosinophil and neutrophil cells as well monocytes. Monocytes differentiate further into macrophages and monocytic dendritic cells (mDC) (Figure 1). These cells provide the basis of immunity that will be discussed in the following pages(1).

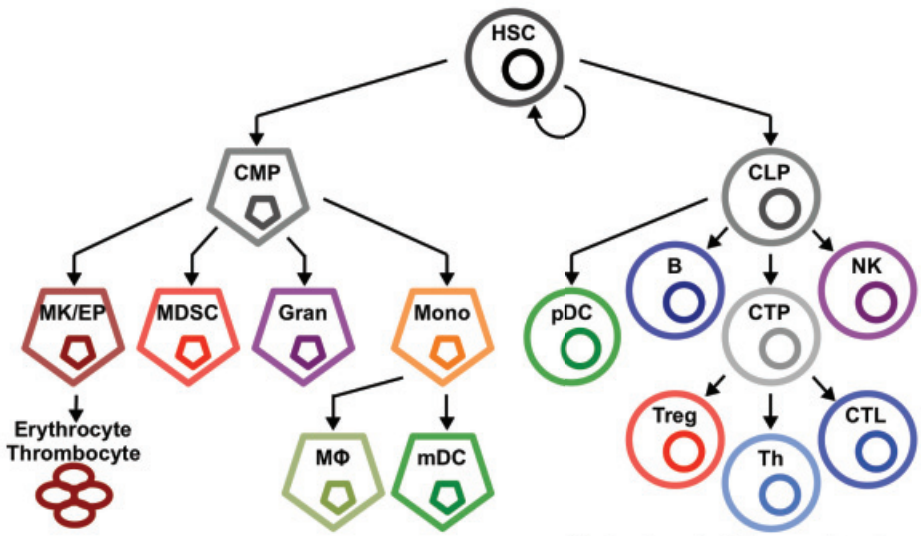


Figure 1. Hematopoiesis is driven by hematopoietic stem cells (HSC) which have the potential to renew themselves or differentiate into the common myeloid progenitor (CMP) or the common lymphocyte progenitor (CLP). The CMP can differentiate into megakaryocyte and erythroid progenitor cells (MK/EP), myeloid derived suppressor cells (MDSC), granulocytes (Gran), monocytes (Mono). MK/EP generate thrombocytes and erythrocytes. Monocytes circulate and can differentiate into monocytic dendritic cells (mDC) as well as macrophages (MΦ). On the lymphoid side the CLP differentiate into natural killer cells (NK), B cells, plasmacytoid dendritic cells (pDC) and the common T cell progenitor (CTP). The CTP migrates to the thymus where it differentiates into cytotoxic T lymphocytes (CTL), T helper cells (Th) or T regulatory cells (Treg).

1.1.1 Innate Immunity

Innate immunity is characterized by nature of its presence prior to challenge by pathogens. The first barrier to entry is mechanical, namely the epithelial surfaces,

which through tight junctions simply do not allow entry to pathogens. Epithelial surfaces are also covered with commensal bacteria that are competitors to pathogenic bacteria. Additionally, internal epithelial layers secrete glycoprotein mucins in mucus that prevent bacterial adhesion. The epithelial cells also have the capacity to produce antimicrobial enzymes, such as lysozyme, and peptides, including defensins, histatins and cathelicidins; these are highly evolutionarily conserved and are able to cause bacterial membrane dysfunction and death. Direct lysis of pathogens is also mediated by the complement system that includes molecules that recognize mannose, antibodies or complement proteins that accumulate on the pathogen cell surface. This initiates protein cleavage cascades that release complement peptides that activate inflammation and recruit phagocytic cells. The cleaved complement signals opsonization and also recruitment of the membrane attack complex proteins that are able to directly lyse pathogens(2,3). While these soluble factors are a potent protection, they often require components of cellular immunity to successfully clear the disease.

Innate Cellular Compartment

The innate immune cells are directly responsible for initiating immediate action against pathogens that have colonized the host, mainly through phagocytosis as well as activation and recruitment of additional immune cells. CMP innate immune derived cells include the granulocytes, macrophages and DCs. The largest proportion of circulating leukocytes is comprised of granulocytes, such as neutrophils, eosinophils and basophils and mast cells. These cells are named after their highly granular nature, and are filled with antimicrobial peptides and enzymes. Neutrophils are highly abundant and short lived. Upon activation neutrophils become highly phagocytic, releasing their granules and even generating neutrophil extracellular traps from their own genomic DNA (4). Eosinophils and basophils have been recognized as essential for parasitic protection, but it is fast becoming clear that their role extends beyond this. They express Fc receptors that allow for binding of Ig coated pathogens leading to the release of cytotoxic basic proteins and inflammatory cytokines and to further recruitment of cells (5). Mast cells are infamous for their role in allergic responses, typically through the release of histamine, but also through release of proteases that damage nearby cells. Mast cells reside at the host-environment interface and respond within seconds to pathogens. They induce the activation of nearby DCs and orchestrate increased cellular migration into infected tissues (6). An other essential class of innate immune cell is the macrophage; these are professional phagocytes responsible for clearage of cellular debris. Macrophages are able to present antigens to the adaptive immune system and support their activation through the production of IL-12 and IFN-γ.

These cellular responses that are of myeloid origin are effective at eliminating extracellular pathogens and recruiting further inflammatory responses. The innate immune system also includes NK cells that eliminate host cells that display signs of "stress". These stresses, due to either pathogen infection or malignant transformation, modulate cell surface receptors which results in destruction by NK cells that are able to recognize these altered surface characteristics. NK cells are typically identified by their expression of CD56, CD16 and lack of CD3. In mice identification of NK cells can be done through their NKp46 or NK1.1 cell surface markers. The levels of CD56 delineate their function - $CD56^{high}$ NK cells are responsible for cytokine production while $CD56^{dim}$ are highly cytotoxic. Activation of NK cells occurs through the balance of activating receptors, such as NKG2D, CD16, DNAM-1 and a host of natural cytotoxic receptor, while inhibitory receptors, such as KIR, NKG2A and CD96 down regulate NK activity. The activating receptors bind to stress induced ligands on the target cell and the inhibitory receptors bind to MHC molecules. When there is an abundance of activation relative to inhibitory signals the NK cell releases its cytolytic perforin and granzyme loaded granules or ligate death receptors through expression of TRAIL and FasL (8,9).

Rolf Kiessling is responsible for laying the foundations of a whole field within immunology. Particularly here at the Karolinska Institutet there are many who are directly indebted to the high background in his assays. About this background reactivity he has said: "High background reactivity in a laboratory assay was the bane of my existence! Much to my dismay, the background lysis of YAC-1 cells in the presence of mouse splenocytes remained high and persisted despite my best efforts, even with cells from non-immunized, control mice. By hindsight, it was through sheer serendipity that we chose YAC-1 cells but the choice was fortuitous indeed since even today, I cannot think of any cell that is more exquisitely sensitive to natural killer cell activity." (7)

Cytokines

Cells of the immune system do not function independently. Rather they are in constant communication with each other and their surroundings. The soluble mediators that facilitate this communication are termed cytokines and chemokines. Cytokines and chemokines are produced by a wide variety of cells including leukocytes, endothelial and epithelial cells. They bind to their cognate receptors on immune cells that induce a biological response such as activation, expansion, maturation, migration or suppression. These molecules can typically be categorized as interleukins, interferons and colony stimulating factors. These soluble factors can have effects in an autocrine, paracrine as well as endocrine fashion, making them amenable for use in immunotherapy. Interleukin 2 (IL-2) was the first described cytokine and is a key regulator of T cell proliferation. Its name describes its essential role in the interaction between leukocytes. Type I interferon α (IFN-α) binds to IFN-α receptor and leads to the upregulation of antiviral proteins and activation of DCs to prime T cells for function (10). Recruitment of appropriate cellular responses is

mediated by chemokine and their cognate G-protein-coupled receptors. Inflammatory responses typically include the production of CXCL8, which leads to the recruitment of neutrophils to combat inflammation-inducing pathogens. The stimulation of different populations of immune cells is also mediated through cytokines such as GM-CSF. This colony-stimulating factor drives the production of new granulocytes and monocytes from the bone marrow (1,11).

1.1.2 Bridging Immunity

Innate immunity is able to clear the majority of pathogens infecting the human host, but some pathogens manage to evade elimination and require a more specific response to be raised against it. DCs mediate the transition from a general response to a specific one. They can be subdivided into classical DC (cDC), mDC, pDC and Langerhans cells. Within these subsets DCs can be further categorized based on the expression of cell surface markers as well as tissue location. These subsets are specialized in particular functions and bridge the innate immune response to the adaptive immunity (12,13).

Antigen Processing and Presentation

The central role that DCs play is that of professional antigen presenting cells (APC). DCs phagocytose pathogens as well as other antibody or complement labeled cells and in doing so take them up in lysosomes where they are broken down into constituent proteins. These proteins are then presented to T cells on MHC class II molecules, while intracellular pathogen proteins are degraded in the cytosol and presented on MHC class I to T cells. MHC class I molecules are recognized by $CD8^+$ T cells, while MHC class II molecules are recognized by $CD4^+$ T cells. The peptides that are loaded into MHC class I are typically from the cytosol of the APC. Cytosolic proteins are cleaved by the proteasome into peptide fragments that range from 8-10 amino acids in length that are subsequently transported and processed through transporter associated with antigen processing (TAP) into the endoplasmic reticulum (ER). MHC class I molecules are bound to calnexin in the ER till they bind to the β_2-microglobulin. This allows for peptide-loading complex to associate. One of the proteins in this complex is tapasin that forms a bridge with TAP and facilitates the loading of the short peptide into the cleft on the face of the MHC molecule. This is then exported via the golgi to the cell surface when loaded. MHC class II molecules are loaded in a different manner, relying instead on extra-cellular proteins as a source of peptides. $CD4^+$ T cells bind to MHC class II and recognize MHC class II loaded peptide. Due to the nature of proteins being loaded are involved in the regulation or responses against extracellular targets. MHC class II molecules are produced in the ER and are loaded with the invariant chain to block binding of endogenous peptides. The invariant chain is cleaved in the endosome, leaving an invariant peptide in the MHC class II cleft. Upon fusion of the lysosome with MHC class II endosomes the invariant peptide is discarded and peptides produced in the

lysosome of lengths varying from 14-20 amino acids are loaded into the cleft. MHC class II is then brought to the cell surface allowing for interaction with $CD4^+$ T cells (1,12). While this describes the classical loading of epitopes, APCs are notorious for finding ways around these theoretical boundaries. Cross-presentation is a phenomena whereby APCs phagocytose antigens and instead of being loaded onto MHC II antigens are leaked to the cytosol and processed by the proteasome, passed through TAP into the cleft of MHC class I (14,15). Cross-dressing is another mechanism that APCs use to present MHC class I complexes loaded by other cells. Here DCs trogocytose MHC class I complexes from adjacent cells and are able to prime $CD8^+$ T cells (16). Additionally, the field of antigen presentation seems to become more complex as it is now clear that alternative pathways of loading epitopes onto MHC are also available to non-APCs including tumors (17).

Activation of Dendritic Cells

Presentation of antigens on MHC complexes is far from adequate to elicit a cellular immune response in itself. All cells are constantly loading peptides into MHC to allow for screening of potential intracellular pathogenesis. What is required is the activation of the antigen-presenting cell. This is achieved through the recognition of pathogen associated molecular patterns (PAMPs) (18) or danger associated molecular patterns (DAMPs) (19). PAMPs are naturally occurring molecules present in most pathogens such as LPS or CpG motifs in bacterial DNA. These are not specific enough typically for inducing anti-pathogen elimination by innate immunity, but will trigger a cascade within DCs leading to maturation and activation. In addition to PAMPs, DAMPs are also able to strongly activate DCs. The distinction between the two is that DAMPs are host derived. DAMPs characteristically are normal cellular molecules that are expressed ectopically in a cell under stress. A good example of this is genomic extracellular DNA, which is normally sequestered in the nucleus (20).

APCs, as well as many other cells, express pattern recognition receptors (PRRs) that bind these PAMPs and DAMPs. PRRs are both transmembrane as well as cytoplasmic receptors that lead to the activation of transcriptional signaling pathways such as NF-κB, activator protein 1 (AP-1) and interferon regulatory factors (IRF). PRRs can also trigger the formation of inflammasomes that lead to the generation of IL-1β and IL-18. These are highly conserved proteins and have been found in all multicellular organisms. The classic family of PRRs are the Toll-like receptors (TLRs), which are similar in structure to drosophila Toll protein, of which there are nine in humans. These bind PAMPs such as flagellin, lipoprotein, peptidoglycans, LPS, dsRNA, ssRNA as well as CpG-rich bacterial DNA which trigger the cytosolic Toll-interleukin receptor domains that signal to adaptor proteins such as MyD88 further propagating a cascade of inflammation. Recognition of these agonists occur both at the plasma membrane as well as intracellularly (21). TLRs do not function individually; co-receptors, such as HMGB1, LL-37 or CD14, may be expressed by

APCs or other activated innate immune cells that increase the affinity of PAMPs for their receptors leading to stronger activation (22).

A relatively recently described class of PRRs are those that sense nucleic acids in the cytosol. This has proven to be an essential mechanism of protection from cytomegalovirus (CMV), herpes simplex virus (HSV) among others (23-25). As well as detecting and activating response against pathogen derived DNA; natural occurring nucleic acids are location restricted, allowing cytosolic sensors for self nucleic acids to be a potent recognition mechanism of cellular dysfunction. DNA binding cytosolic sensors includes: DAI, LRRFIP, AIM2, IFI16, cGAS and many more. There is extensive redundancy within that hints at the essential nature of these molecules. Due to the wide variety of sensors the localization is still under investigation. As with the classical PRRs these cytosolic DNA sensors lead to the

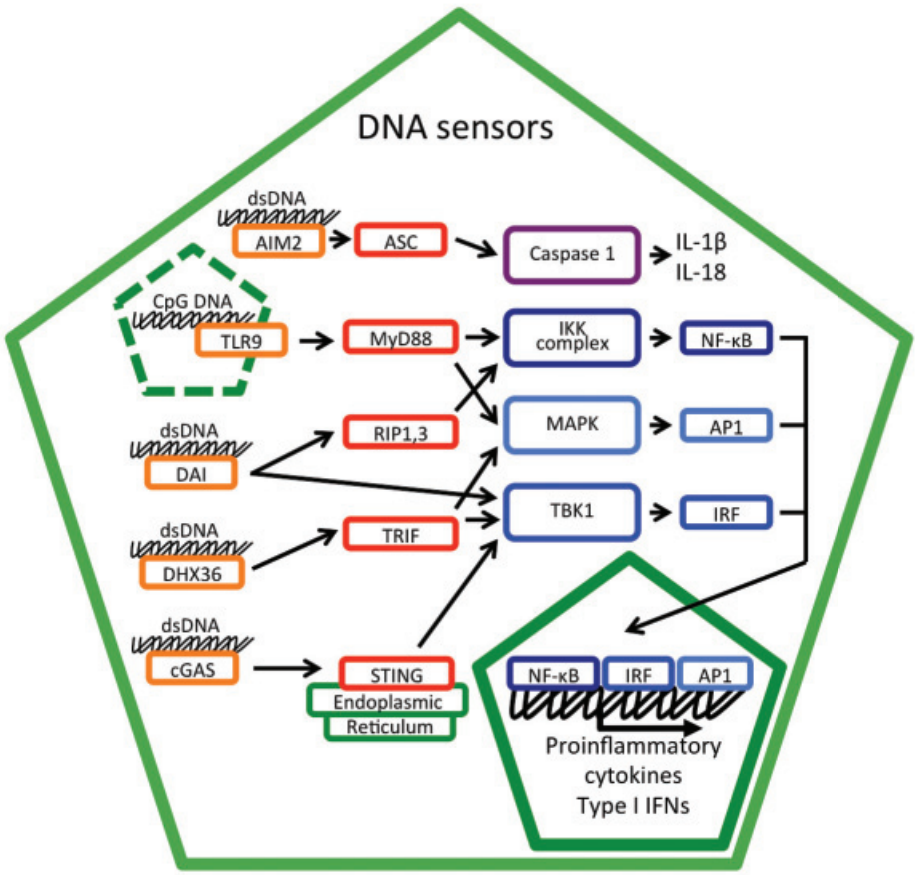


Figure 2. DNA sensors, including but not limited to AIM2, TLR9, DAI, DHX36 and cGAS activate transcription factors NF-κB, AP1 and IRF through IKK, MAPK and protease Caspase 1 to initiate pro-inflammatory and antiviral responses. These sensors mediate their function through the adaptor protein complexes ASC, MyD88, RIP1 and 3, TRIF and STING.

activation of transcription factors AP-1, NF-κB as well as IRFs. AP-1 and NF-κB are responsible for the up-regulation of survival, migration, maturation and pro-inflammatory genes. While there is such a broad panel of molecules directly binding to DNA, the activation of IRFs seem to converge on the stimulator of interferon genes (STING) adaptor protein (Figure. 2) (26-28). Cytosolic DNA through these sensors is capable of strong activation of DC leading to signaling through all three essential signals for T cell activation.

The Three Signals for T cell Activation

When the processed peptides are presented and the DCs have become activated through PRR activation by DAMPs or PAMPs then the scene is set for inducing resting naive T cells to a state of activation and clonal proliferation. DCs, that are activated, migrate to the secondary lymphoid tissues and increase their capacity to present antigens. This is the first signal. The second signal is the upregulation of co-stimulatory molecules. These are proteins of such as CD80, CD86, ICOSL and CD40 on DCs that bind to the CD28 family member proteins on T cells. Finally, the third signal comes in the form of cytokines produced either directly by the engaging DC or from peripheral signaling. Specific cocktails of interleukins sensed by T cells will shape the response generated. IL-12 and type I IFNs provide a means for the encountering $CD8^+$ T cell to sense 'danger' (29,30). Together, these three signals are required for activating cellular responses. When one is missing, this can lead to T cell anergy or even skewing T cells towards a regulatory phenotype.

1.1.3 Adaptive Immunity

Although the components of adaptive immunity have been mentioned above, adaptive immunity, as the name implies, describes the ability of the arm of immunity to adapt a specific response towards pathogens evading innate immune elimination. The incredible capacity for the adaptive immunity to respond to pathogens with an individualized approach lies in their ability to rearrange genes that encode for different parts of either their T cell receptor (TCR) or B cell receptor (BCR) (1). T cells derive their name from their trafficking to the thymus prior to entering circulation. T cells come in two flavors, namely CD4 and CD8, which can further be segregated. The first has classically been termed helper T cells and are essential in the production of cytokines and other factors to regulate and establish immunity. They bind epitopes presented in MHC class II and are essential to B cell maturation into antibody producing plasma cells as well as being essential to produce cytokines to give the "third" signal to activate other T cells. The CD8 proteins on T cells allow them to bind to MHC class I, recognizing the epitope presented triggers a cascade that typically leads to lysis of target cells. B cells derive their name from their origin of discovery, namely the bursa of Fabricius in birds that coincidently is also appropriate in humans where they originate from the bone marrow. They enter circulation and with their BCR bind their cognate antigen. The B cells then process and present this

antigen to be recognized by T helper cells who then initiate the differentiation of the B cells into plasma cells that produce antigen specific clonal antibodies (1).

1.1.4 Restraining Immunity

The near infinite rearrangements available to adaptive immunity endow it with the ability to react to a nearly endless repertoire of antigens on pathogens. This boundless potential also has a great weakness, i.e. randomization of specificity will also generate responses that have the ability to react to self-antigens. It has been proposed that self-reactive T and B cells are eliminated by central tolerance. Both T and B cells, in their common lymphoid progenitor cell (CLP) state have their full genomes prior to recombination. Pre-T cells migrate to the thymus where, after recombination of their TCR, they undergo positive and negative selection. The first requirement is that the randomly recombined TCR binds MHC. If this is the case, the fate of $CD4^+CD8^+$ pre-T cells is determined to either CD4 or CD8 lineage. In the next phase, negative selection deletes T cells that bind with high affinity to MHC presented "self" antigens by medullary thymic epithelial cells. Pre-B cells expressing surface IgM that bind strongly to cell surface expressed antigens are eliminated in a process termed clonal deletion. In the case that B cell IgM binds to soluble self-antigens they are not deleted but become anergic. If there is no binding they go into circulation, awaiting $CD4^+$ helper T cell activation (31). When in circulation after central tolerance, T cells and B cells encounter peripheral tolerance. In the periphery they may encounter DCs that present their cognate antigen on MHC but lack second or third signals, thus inducing anergy in the cells. Additionally, regulatory T (Treg) cells mediate suppression via the production of inhibitory cytokines, suppression of DC maturation and function. Thus preventing T or B cells from activating (32) and providing the basis for the "self-non-self" theory.

Peter Medawar and **Macfarlane Burnet** were instrumental (and won a Nobel Prize) for developing the theory of immunological tolerance. Peter spent a large amount of time during the Second World War with the problem of rejected skin transplants in burn patients. One burn patient "Mrs. McK" was treated with both her own transplanted skin and that of her bother. Medawar observed that her brothers skin was rejected, and rejected more rapidly the second time around while her own was not. This started Medawar on the road to understanding transplantation and acquired tolerance. Medawar was responsible for executing the experiments that Macfarlane suggested would be the proofs for establishing the theory of immunological tolerance. He suggested about acquired tolerance: "If in embryonic life expendable cells from a genetically distinct race are implanted and established, no antibody response should develop against the foreign cell antigen when the animal takes on independent existence". (33)

While central and peripheral tolerance are well-established mechanisms they do not determine the entirety of immunological regulation. An alternate theory of tolerance has been proposed, and discussed a bit above in the form of DAMPs, namely the "danger theory" suggests that "self-non-self" T cells are not restricted but await DC

activation through DAMPs to stimulate responses (19,34). Additionally, tolerance could be mediated differently in each tissue-based location (35). It seems to be more of an amalgamation of these theories as recently Yu *et al.* has been able to experimentally substantiate theories that central tolerance deletes self-reacting T cells but only to a limited degree. They found that in the periphery of non-infected donors the circulation of both foreign epitope specific T cells as well as a large amount of endogenous epitope specific T cells, related to keratin, preproinsulin and aldolase, were present. When evaluating a Y chromosome encoded antigen and comparing female to male healthy donors, only a reduction of 33% was observed (36) indicating that self-reactive T cells were indeed present and could be potentially primed.

1.2 CANCER IMMUNOLOGY

Cancer is a disease of unrestricted proliferation of cells. The uncontrolled nature of cell growth by cancer can be caused by many factors, such as mutations in oncogenes and tumor suppressor genes as well as virally encoded proteins causing deregulation of the cell cycle. Typically two to eight driver mutations are required to initiate tumorigenesis, a varying fraction of which eventually develop into metastatic cancer. Classic mutations occur in KRAS, SMAD4 and TP53, and have been very well documented in colorectal carcinoma (CRC). These driver mutations fall into three categories that regulate cellular processes, namely: genome maintenance, cell fate and cell survival. The accumulation of these mutations that lead to tumorigenesis can take decades (37,38). The single cell that acquires the mutations to initiate tumorigenesis does so due to exposure from endogenous as well as exogenous factors. These factors include oxidative stress induced by cellular metabolism, radiation, carcinogens and cellular machinery errors resulting in DNA strand breaks (39). These errors can be detected and directly repaired by inherent DNA repair mechanisms or indirectly through the activation of apoptosis. The accumulation of mutations in oncogenes or tumor suppressor genes, such as those mentioned above, lead to tumorigenesis which endows the mutation-rich cell with the hallmarks typically present in cancer. These include, sustained proliferation, resistance to cell death, angiogenesis, evasion of growth suppressors, replicative immortality, genomic instability, escape from immune destruction, tumor-promoting inflammation, deregulated cellular energetics as well as invasive characteristics (40). This is quite an extensive list, and for the tumor to become an aggressive cancer it is required to fulfill these hallmarks. This may explain why the frequency of cancer is not overwhelming in the human population.

1.2.1 Spontaneous Anti-Tumor Immunity

In the previous sections we have been referring to pathogens and their interaction with the immune system. I would like to draw your attention to the fact that in most aspects the hallmarks of cancer are exceedingly similar to those we would use to

describe a successful pathogen. The major distinction of course is that most cancers are not directly transmittable (with the exception of intentionally transplantable tumor models, certain canine malignancies and Tasmanian devils facial tumors (41)). The importance of immune mediated tumor surveillance is now well established after struggling for years with the concepts of “self-non-self” tolerance and inadequate tools to study this interaction.

The first clear indications of the importance and involvement of immune rejection of tumors was found when the technology became available to transplant organs from donors into recipients. This is one of the greatest kindnesses possible for us to bestow upon our fellow human, the irony is immense when this blessing turns into a life-threatening curse. For example, a kidney transplant was done on a patient with polycystic disease that after engraftment functioned well. After discovery of a nodule in the breast a biopsy was performed and was diagnosed as a secondary melanoma, no primary melanoma could be found. The patient had been on immunosuppressants, which were stopped immediately, but to no avail, she died of metastatic melanoma. The recipient of the other donor kidney was found to also have metastatic melanoma. Fortunately, this was restricted to the donated organ. After halting immunosuppression and treatment with interferon the kidney was rejected along with the tumor. The donor had a melanoma removed 16 years prior to donating the organs and being tumor free for 15 years (42). Multiple follow-up studies on renal transplants have shown significantly increased incidence of cancer in these patients compared to the predicted frequency in the general population (43,44). Cardiac transplants have similar side effects with patients receiving immunosuppressives to retain the graft, but allowing for escape of the tumor in the donor tissue from immunosurveillance. In cardiac and lung transplant patients, lung cancer derived from the transplant led to extremely poor prognosis (45,46). Additionally, these studies indicate that there are two categories of tumorigenesis: those driven by virus and those driven by mutational burden. Virally induced cancers are much more likely to occur in immunosuppressed patients (47). This was also the case when immunosuppression was caused by HIV/AIDS (48).

The second proof arose from the development of monoclonal antibodies reactive to a wide variety of immune related antigens. Once this was available, and paraffin embedded patient tumor samples were accessible initially for immunohistochemistry, and presently augmented by flow cytometry, the race was on to search for and identify prognostic markers that were immune related. Many research groups were able to identify that $CD8^+$ cells were prognostic in melanoma, head and neck cancer, breast cancer, renal cell carcinoma, colorectal carcinoma and non-small cell lung cancer among many others (49). It was the evaluation of CRC and the immunoinfiltrate that conclusively showed that T cell infiltration was key to improved prognosis. This was done by Galon *et al.* where they were able to show that CD45RO and CD3 in the invasive margin as well as in the central tumor predicted

disease-free survival. Establishing an immune contexture and immunoscore they were able to break down the classic UICC TNM staging system into new categories that showed that patients typically found in TNM III could have a 80% disease free survival if they had $CD3^{hi}CD45RO^{hi}$ infiltration of both the invasive margin as well as the center of the tumor (50). Other infiltrating immune cells, such as B (51), NK (52-54) and DCs were prognostic for delayed tumor progression (52).

Other immune cells correlate with a negative prognosis for patients. For example; $CD68^{+}$ tumor associated macrophage (TAM) infiltration is a negative prognostic marker in classic Hodgkin's lymphoma (55). TAMs are recruited to the tumor by CCL2 and result in the progression of colorectal cancer (56). Infiltration of macrophages into invasive breast carcinoma correlated with increased vascular grade and reduced relapse-free survival (57). MDSC predict the survival of metastatic melanoma patients (58). Tregs predict negatively the outcome of colorectal cancer that has spread to the liver (59), but they have also been shown to be a positive prognostic marker (60) potentially due to their recruitment when anti-tumor immune responses generate IL-2 and IFN-γ. The immune landscape of cancer is complex, but a clear fingerprint is being elucidated that will provide robust prognostic and predictive benefit for patients (61).

The final group of evidence is based upon the carcinogen-induced tumors in various mouse models. Early on, it was described that carcinogens, such as methylcholanthrene (MCA), could induce sarcomas, particularly in inbred strains of mice (62). These MCA induced tumors were shown to induce immunity against specific tumors but not others. Furthermore, mice that had a primary tumor regress were immune to tumor growth when rechallenged with the same tumor but not others (63).This was followed up by vaccination studies that clearly demonstrated the immunogenicity of carcinogen-induced tumors (64). Later is was confirmed that $CD8^{+}$ T cells were responsible for recognizing MCA sarcoma antigens (65). Using the MCA sarcoma model, which was know to generate immunogenic tumors, and nude mice Engel *et al.* was able to show that mice deficient in thymus, and there for T cell development, were more susceptible to MCA induced tumors (66). Similar

Paul Ehrlich's contributions to the field of immunology were fundamental in nature. He postulated that cells were responsible for the production of chemical structures, "side-chains", that would bind toxins. The side chain would then break off the cell and enter circulation; seeking out and neutralizing toxins as "magic bullets". His depictions of these "side-chains" are remarkable similar to how antibodies are represented to date. In addition to this Paul speculated that these antitoxin responses could play a role in immune surveillance. He said: "I am convinced that during development and growth malignant cells arise extremely frequently, but in the majority of people they remain latent due to the protective action of the host. I am also convinced that this natural immunity is not due to the presence of antimicrobial bodies but is determined purely by cellular factors. These may be weakened in older age groups where cancer is more prevalent." (68)

results were found in Rag2 knock out (KO) mice, a more restricted mouse model of T cell deficiency (67). One of the main mechanisms of T and NK cell lytic capacity is dependent on perforin. MCA induced sarcomas, virus induced tumors and spontaneous lymphomas were studied in perforin KO mice. In both instances the tumors grew rapidly in KO mice but not in wild type (WT) mice (69-71). Further investigation into immunosurveillance was done using IFN-γ receptor KO mice, which were more susceptible to MCA induced sarcomas than their WT litter mates (72). Not only were NK and T cells implicated in immunosurveillance, but also γδ T cells are responsible for surveillance via NKG2D (73). Type I interferon were also designated a role in immunosurveillance (74). Finally, on the other side of the spectrum, depleting T regulatory cells allowed for enhanced immunosurveillance (75).

All three aspects come together to form definitive proof that immunoediting is ongoing during tumorigenesis. This model consists of three potential states, whereby initially the tumor is eliminated. This elimination is mediated by any of the above-described cytolytic mechanisms. If the tumor manages to evade direct elimination it may enter a stage of equilibrium where there is a constant tug of war between anti-tumor immunity and tumor growth. This was definitely the case for the unfortunate patients who received the metastatic melanoma infested kidneys whose donor had no signs of disease for 16 years. Finally, the tumor enters a state of escape. Escape leads to the dissemination of the disease throughout the host, and is typically when the patient enters the care of the medical system (Figure 3) (76). The mechanisms of escape are described below.

1.2.2 Tumor Immune Escape Mechanisms

Experiments proving the importance of immune-mediated tumor elimination and equilibrium have established that the tumor must escape the cytolytic anti-tumor responses of various populations of lymphocytes for clinical disease to occur. The method with which this happens is not merely through the selection of immunoevading tumor clones. While the majority of tumor variants are eliminated, few clones with the ability to avoid immune detection and/or possessing mechanisms to actively suppress the immune system manage to establish themselves and proliferate into clinically obvious disease. Tumors of substantial volume gain additional immunoevading features as well as recruiting immunosuppressive cells into their milieu. An example of the immunosuppression induced by tumors that has been described in a wide variety of tumors, including but not limited to RCC (77), CRC (78), oral carcinoma (79), is where T cells lose CD3-ζ chain and thus their ability to function.

1.2.2.1 Tumor derived immune evasion

Reducing detection of tumors by T cells is one of the mechanisms that tumors employ to evade elimination. Expression of MHC class I was found to be downregulated or deregulated in several tumor types. Alterations in MHC class I are often observed to be due to direct structural alterations or transcriptional or post-transcriptional dysregulation. Mutations in the HLA class I β_2 side chain is mutated in 21% of CRC and 15% of melanomas. Haplotype loss was found in 36% of head and neck squamous cell carcinomas. These modifications allow for evasion for immune surveillance (80). In addition to loss of MHC, tumor antigens can be lost, shielding tumors from TAA specific T or B cell responses. Additionally, tumors equip themselves with increased levels of antiapoptotic proteins such as survivin and cFLIP that are able to render the tumors resistant to death receptor-mediated apoptosis. While impervious to death receptor-mediated killing themselves, tumors exploit these mechanisms and express FasL which induces death in Fas^+ immune cells. FasL expression has been described in melanoma, colon carcinoma and lung carcinoma (81). Tumors also present PD-1 ligands (PD-L1 and PD-L2) that induce anergy in T cells directly after contact. In addition to direct cell interaction molecules, tumors upregulate immune suppressive and tumor promoting cytokines such as VEGF, IL-10, TGF-β, PGE2, soluble Fas and FasL (82). Finally, solid tumors are notorious for creating oxidative stress (83) that directly impact the survival of anti-tumor immunity (84) but are able to enrich suppressive Tregs due to their increased TRX1 levels (85) .

1.2.2.2 Tumor recruited immune suppressive cells

The chronic inflammation that the tumors induce leads to the recruitment of diverse cells types whose principle function is to dampen inflammation. In a natural pathogenic event, the immune system responds and induces inflammation to allow for the recruitment of a wide variety of cells to clear the pathogen. Once this has been achieved and inflammation is no longer required Tregs and Myeloid-derived suppressor cells (MDSC) are recruited to subside the immune response. This mechanism is hijacked by the tumor to facilitate its immune escape.

Tregs have an essential role in peripheral tolerance, and when recruited to the tumor microenvironment are able to significantly inhibit anti-tumor immune responses. Tregs are $CD4^+$ T cells typically characterized with the high expression of CD25 and FoxP3. They have a myriad of different methods by which they exert their suppressive nature. Tregs, when activated, secrete IL-10, TGF-β and IL-35 that bind to their cognate receptors on conventional T cells that suppress cytokine production and cytolytic as well proliferative functions. IL-2 is required for T cell activation and proliferation; the high expression of CD25 facilitates the ability of Tregs to remove all local IL-2. This effectively blocks second signals from reaching anti-tumor immune

cells. In addition to mopping up IL-2 from the immediate milieu, Tregs are able to quench the local environment of ATP, generating adenosine that in addition to inhibiting T cells is able to tolerize DCs. Tolerizing DCs is the other main mechanism that Tregs utilize to prevent further T cell activation. It has been proposed that Tregs express LAG3 to bind MHC class II molecules and activate ITAMs within the DCs (32).

MDSC are recruited to the tumor microenvironment by chemokines as well as other tumor-secreted factors such as IL-1β, CSF and PGE2. The population of cells termed MDSCs are very well defined in mice, namely they express CD11b and Gr1 cell surface proteins and are suppressive in nature. In humans the term MDSC refers to a highly heterogeneous population that has a variety of cell surface markers associated with them, but the key factor is their ability to be immune suppressive. Their suppressive potential is derived from multiple mechanisms. The secretion of suppressive cytokines, similar to those produced by Tregs, IL-10 and TGF-β. In addition to cytokines MDSC are able to produce ARG1 that catabolizes L-arginine to urea and ornithine, limiting the access of this essential amino acid to proliferating T cells. MDSC also deplete L-arginine from the microenvironment by producing iNOS that converts L-arginine to citrullin and nitric oxide. NO is typically converted into radical peroxynitrite, which can lead to the nitrosylation of TCRs, impairing their function. Reactive oxygen species are another potent mediator of immune dysfunction. NADPH oxidase subunits generate super oxide upon MDSC activation and mediate suppression in a cell-cell contact dependent manner (86). In addition to these mechanisms prostaglandins function directly as suppressive signals to effector T cells as well as supporting further recruitment and generation of MDSC (87).

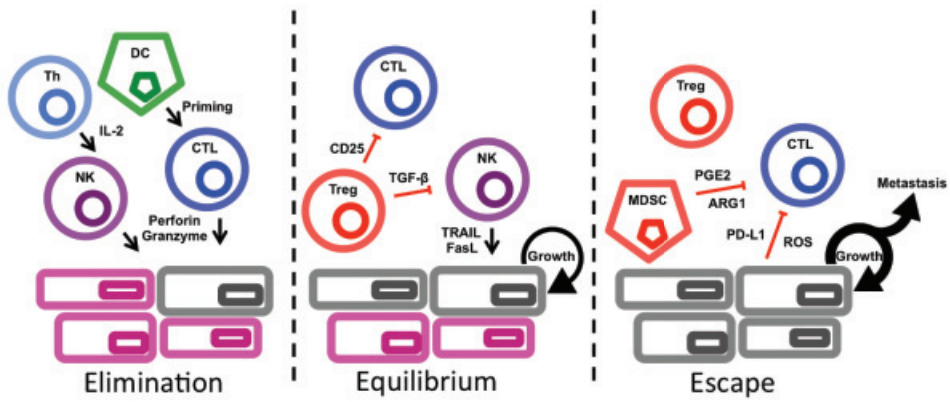


Figure 3. The three Es of tumor immunosurveillance are represented by the elimination of the tumor cells (grey) but not healthy cells (pink) by NK and CTLs that are assisted and primed by Th and DCs. The tumor growth may reach equilibrium with immune mediated killing, mediated in part by TRAIL and FasL expression on NK cells. Chronic inflammation leads to the recruitment of suppressive immune cells such as Tregs that suppress T and NK cells by expression of TGF-β and removing IL-2 from the microenvironment by high expression of CD25. Escape occurs when tumor growth is accelerated beyond the killing capacity of antitumor immunity. This is mediated in part by the accumulation of reactive oxygen species (ROS) that induce oxidative stress as well as high expression of PDL1 on the tumor cells. Additional suppressive cells are recruited, such as MDSC, that secrete ARG1 and PGE2. This allows the tumor to grow rapidly and metastasize.

1.2.3 Tumor Antigens

Anti-tumor immunity can be mediated through various pathways, as described above. Some of the most potent are those mediated by adaptive immune cells. The ability of adaptive immune cells to mediate tumor killing requires the recognition of tumor antigens. There are two classes of tumor antigens, those that are unique to the tumor and those that are shared with healthy tissue.

Antigens that are unique to the tumor are prime targets for anti-tumor immunity due to the fact that there can be no central tolerance to these antigens and TCR as well as BCR affinities to those epitopes can be high. Tumors that are driven by mutations have a unique immunopeptidome, and these mutations can generate novel epitopes by altering a specific amino acid, shifting the reading frame or by extending the protein beyond the stop codon. The T cell response against melanoma is dominated by neo-antigen specificity (88). The anti-tumor immune response, and the prognosis for the patient, is determined by the immunogenicity of the mutations. If more than one immunogenic mutation is present the survival probability of the patient is improved (89). Tumors that are driven by viral infection, such as: head and neck, cervical, adult T cell leukemia, hairy-cell leukemia and Kaposi's sarcoma all have the possibility to present highly immunogenic viral antigens. These antigens are unique to the virus and prophylactic vaccination against HPV, the driver virus behind head and neck as well as cervical cancer, has already proven effective. Gardasil and Cervarix, vaccines specific for HPV-16 and 18, induce high levels of antibody that neutralize HPV. HPV specific T cell epitopes have also been identified and are being used as targets for therapeutic vaccination (90). Tumors of the hematological B cell variety also express a unique antigen. These malignancies have, if their BCR is fully rearranged, a unique variable region in their BCR that defines is specificity, the idiotype (Id). There is a natural anti-Id antibody response that occurs upon high levels of immunoglobulin production, revealing the lack of tolerance to these antigens. Unique tumor antigens represent an interesting class of targetable antigens, particularly the viral antigens. The neo-antigen and Id-antigens retain the requirement of a per-patient identification of the epitopes and equally unique immunotherapeutic approaches (91,92).

The other class of tumor antigens are those shared by the tumor and normal healthy tissue. But within this class there is a type of tumor antigen that is relatively specific; these are the cancer germline antigens (also known as cancer testis antigens). These are antigens that are expressed in relatively exclusively to testicular and ovarian germ cells. The MAGE family of cancer germline antigens have been found to be expressed in many different cancer types, including breast cancer, CRC and melanoma (93). NY-ESO-1 is a cancer testis antigen that was identified through screening of serum for cancer patients on bacteria transformed with tumor cDNA.

NY-ESO-1 is expressed by a wide variety of tumor types and represents an interesting tumor antigen due in part to its cell surface expression, allowing for antibody therapy, as well having described T cell epitopes, allowing for T cell therapy (94). Differentiation antigens represent a group of TAA that are antigens shared with a specific differentiated cell type. The most common examples are MART1, tyrosinase and gp100 that are all antigens expressed on melanocytes as well as melanomas. Targeting these antigens has shown to be effective, but has led to on-target off-tumor side effects such as vitiligo. MART1 is an interesting antigen due to its relatively high frequency of specific T cells, 1 per 10^3, in healthy donor PBMC. Finally, the least specific TAA are those that are overexpressed on tumors, but are also expressed on healthy tissue. HER2, for example, is a member of the human epidermal growth factor receptor family and can be over expressed on breast cancer. Current antibody therapies target this molecule, but HER2 is also expressed on the cardiomyocytes epithelial cells and other tissues (95,96). Other tumor antigens overexpressed in tumors are carcinoembryonic antigen, Wilms' tumor protein (WT1), which is expressed 10 to 1,000 fold higher in leukemic cells, and mucin 1 (MUC1), which is often overexpressed on adenocarcinomas and has both T as well as B cell epitopes (91,92,97).

2 PILLARS OF IMMUNOTHERAPY

The pillars of cancer immunotherapy can be divided into two types, namely those therapies that can be either classified as passive or active immunotherapy.

2.1 PASSIVE IMMUNOTHERAPY

Passive immunotherapy includes those pillars of immunotherapy that are built around introducing immune system components, such as cytokines, monoclonal antibodies or adoptively transferred cells, into the patient that provides immunity against the tumor.

William B. Coley is the grandfather of cancer immunotherapy. Serendipitously, he noticed that patients with sarcomas could have regression of their disease after severe infection post surgery (at this time, sterile technique was a novel concept). He decided to intentionally infect sarcoma patients with *Streptococcus pyogenes;* this immediately led to difficulties in dosage, infection rate and sepsis related fatality. To make patient responses more uniform to treatment he switched to an inoculation with killed *S. pyogenes* and *Serratia marcescens*. His first patient treated with this inoculation had a strong response that led to total disease regression; the patient died 26 years later of a heart attack. “Coley’s toxins” were used many different malignant diseases with some success, but few studies were poorly documented, and with the advent of chemo and radiation therapy “Coley’s toxins” fell into obscurity. (99)

2.1.1 Cytokine Therapy

Cytokine-mediated signaling between cells cumulatively results in a diverse and pathogen-specific response. Cytokines, the small molecules responsible for intracellular communication, were one of the first immunotherapeutics available for the therapy of cancer patients. It is exactly for their role in activating and stimulating particular immune cell compartments that they make for interesting molecules to deliver. Two type I interferon molecules have been approved by the FDA. These are IFN-α2a, used in patients with hairy cell leukemia and Philadelphia chromosome-positive CML, and IFN-α2b which is applied to diseases such as AIDS-related Kaposi’s sarcoma, follicular lymphoma, melanoma and cervical cancer. The FDA has also approved IL-2 for the treatment of metastatic melanoma and renal cell carcinoma. Many other cytokines are used in combination with varying immunotherapeutic modalities (98).

2.1.2 Antibody Therapy

Antibody therapy became a reality once the technique for the generation of monoclonal antibodies had been properly harnessed. In 1975 the first monoclonals were generated using hybridoma techniques with the first licensed monoclonal being OKT3, the antibody binding to CD3, for treatment in preventing kidney transplant rejection (100). Monoclonal antibodies are able to mediate an anti-tumor effect by binding directly to tumor antigens, or by engaging antigens found in the tumor microenvironment that indirectly lead to an anti-tumor effect. Antibody therapies have a couple of functional modalities, which include: Fc-region activation of

cytotoxicity through ADCC, ADCP and CDC, the inhibition of target protein signaling, the activation of TRAILR2 or other apoptosis inducing receptors, the delivery of toxins or radionuclides, the binding of factors produced by the tumor to enable growth and metastasis (101). Unconjugated antibodies used for therapy include Rituximab, which binds to CD20 expressed by non-Hodgkin lymphoma and chronic lymphocytic leukemia (102), Trastuzumab, which binds to HER2 on breast cancer (103,104), Cetuximab, which binds to EGFR on colorectal cancer (105) which have human IgG1 Fc regions allowing for functional Fc based cytotoxicity. These monoclonal antibodies target the tumor directly, though there are also a plethora of antibodies targeting the vast array of tumor associated pathways (106).

Recently, antibody based therapy has once more come into the spotlight through the effective targeting of immune related targets, of which inhibitory checkpoint receptors PD-1 and CTLA4 targeted antibodies are having a phenomenal impact on cancer therapy. These antibodies block the inhibition of activated T cells. Ipilimumab and Tremelimumab, both target CTLA4 expressed on activated T cells and interfere with engagement of the ligands CD80 and CD86 on APC. Pembrolizumab and Nivolumab, both bind PD-1 and block its interaction with its cognate ligands PD-L1 and PD-L2 expressed on a wide variety of cells including tumors (107,108).

CTLA-4 blockade in stage III or IV melanoma increased overall survival to 10 months compared to 6.4 months for patients receiving gp100 peptide vaccines. This was a surprising finding as the group with gp100 plus Ipilimumab had similar overall survival to those only treated with Ipilimumab (109). PD-1 blockade using Nivolumab was able to induce objective responses in one of four patients burdened with either non-small-cell lung cancer, melanoma or renal-cell carcinoma but not in castration resistant prostate cancer or colorectal cancer (108,110,111).

Monotherapy with checkpoint inhibitors has yielded promising results, pushing researchers to dream of combination therapies. Of course, as with all combination therapies, there are nearly unlimited combinations possible, but there are a couple I would like to highlight. Checkpoint blockade therapies could be combined together. Combination of Nivolumab together with Ipilimumab resulted in objective-response rates of 40%, with 65% of patients having immune-related clinical activity (112). This may not be surprising as both pathways provide T cell checkpoint blockades at different stages. The synergy between these antibodies and vaccination strategies is also very apparent. One example is the combination of αPD-1 treated mice together with multi-peptide based vaccine in a breast cancer mouse model (113). Combinations with checkpoint blockade antibodies also extend to classical therapies, where fractionated radiotherapy delivered in combination with either αPD-1 or αPD-L1 increased tumor control in a IFN-γ T cell dependent manner (114). This was also the case of radiotherapy combined with anti-CTLA-4 antibody (115,116).

Additionally, the exploration of chemotherapies together with checkpoint inhibitors may prove fruitful (117,118).

Patients who benefited from CTLA-4 blockade treatment were demonstrated to have high mutational load in their primary tumors. Further evaluation established that a neo-epitope signature could be predictive towards response to CTLA-4 treatment (119). This observation potentially extends towards other checkpoint inhibitors as well as immunostimulatory antibody therapies. Patients with colorectal carcinoma whose tumors lack the ability to correct mismatch defects have been reported to have greater benefit with anti-PD-1 therapy with significantly increased overall survival, a response pattern akin to that noted with neo-antigens and CTLA-4 blockade (120). The above observations highlight the importance of T cell specific responses to unique tumor antigens.

2.1.3 Adoptive Cell Therapy

Originally, cancer therapy using cell transfer was done to restore the immune compartment in patients receiving high-dose myeloablative treatment. Myeloablative therapy could effectively cure some patients, but would result in hematopoietic failure. This led to attempts to rescue with hematopoietic stem cell transplant (HSCT) after treatment. HSCT in turn came with graft versus host disease (GVHD), the immunological rejection of host cells by grafted immune cells, which in some cases had a beneficial effect associated with lower relapse rates due to graft versus tumor effects. This graft versus tumor effect leading to potential cures in some patients was the first indication that immunotherapy using adoptive cell therapy (ACT) could be a potent actor in cancer therapy (121,122).

2.1.3.1 Tumor infiltrating lymphocytes

The transplant of HSC into patients after chemotherapies always had, and still struggles with severe GVHD. This undesirable off-target effect can be ameliorated by harnessing the antigen specificity of T cells. The difficulty however is that T cells specific for the tumor must be found in the patient. T cell infiltration into tumors is in most instances, a good prognostic factor for many tumor types. Tumor infiltrating lymphocytes (TIL) represent a source of tumor reactive T cells without the required exploration for patient-specific tumor antigens. Tumor samples are excised from the patient, cut into pieces and cultured with high dose IL-2. This leads to lymphocytes overgrowing the tumor tissue and allowing for testing of tumor reactivity. Tumor infiltrating lymphocytes are then expanded to approximately 10^{10} cells and re-infused into patients. Originally tumor-reactive TIL were generated from MC-38 colon adenocarcinomas in mice. Syngeneic mice treated with TIL in combination with IL-2 and cyclophosphamide had dramatic increase in survival (123). Within two years this therapy, without the cyclophosphamide, was applied in human patients bearing resectable metastatic melanoma lesions, with regression in observable tumors in

60% of patients (124). When cyclophosphamide was added to the treatment regimen no difference in response rate was found between the arm without or with cyclophosphamide, while objective response rate was around 30% (125). This was followed up by using a total body irradiation, either chemotherapy alone, two or 12 Gy irradiation, to lymphodeplete the patients prior to ACT. In the arm receiving the highest irradiation 72% of patients had objective response compared to 49% in the no total body irradiation group (126). These reports of TIL therapy of melanoma, driven in large part by the Rosenberg group at the NIH, has now spread to many other research institutions, including the Karolinska Institutet.

TIL therapy requires the generation of tumor reactive lymphocytes derived from the patients tumor, this is frequently not possible due to surgical specimens not being available or limited infiltration of lesions by lymphocytes. Oved K *et. al.* has been able to establish that within non-reactive TIL there are populations of tumor reactive T cells. They generated an algorithm-based approach for enriching for tumor reactive TIL by screening patients that produced reactive TIL and compared their immunological signature with patients that had non-reactive TIL. To test their approach new patients were recruited, and from the 12 non-reactive TIL it was possible to recover 9 that would qualify for inclusion in TIL ACT (127). Removing non-essential, potentially suppressive cells from non-reactive TIL can be combined with positive selection for PD-1 positive TIL. T cells from TIL that were positive for inhibitory molecules, PD-1, LAG3 and TIM3 positive, were identified to be responsible for the autologous tumor reactive repertoire found in TIL (128). Though these strategies do increase the potential patient pool for TIL therapy, there will be many diseases for which this strategy will remain unfeasible (129,130).

2.1.3.2 Transduced T cells

The T cell receptor, and random recombination of its genes, enables T cells to specifically target antigens. TIL can be reactive towards tumor specific antigens, particularly those that have generated de novo mutations termed neo-antigens (131). In those patients where TIL and tumor specific T cells cannot be generated it is possible to engineer T cells with TCRs specific for tumor specific antigens. TCRs specific for MART-1, a melanoma differentiation antigen that has been a model melanoma tumor associated antigen, had been cloned out of human lymphocytes and transduced into patient PBL. MART-1-TCR-T were able to specifically react to MART-1 positive tumors in a HLA-A2 dependent manner. Two patients out of 15 had objective responses (132). An antigen that may prove interesting for tumor specific TCR redirected T cells is NY-ESO1. This is a cancer testis antigen that is well characterized, being expressed on 80% synovial cell sarcoma as well as 25% of melanomas. Treatment of patients with NY-ESO1-TCR-T led to four out of six objective responses in synovial cell sarcoma and five out of 11 objective responses in melanoma (133). To circumvent the pruning of high affinity TCRs by central

tolerance mouse TCRs have been isolated in HLA-A2 transgenic mice specific for an epitope shared between MAGE family antigens. When MAGE-TCR-T cells were transferred into patients, there were objective responses against the target as well as serious adverse events leading to death in two patients. The TCR transduced T cells recognized MAGE-A12 in brain tissue (134). Further experimentation with modified MAGE-TCR-T led to severe cardiac toxicity and additional morbidity (135,136). This shows the extremely potent nature of redirecting T cells against antigens not well defined.

Transducing T cells with designer genes provides a powerful tool to generate T cells with tumor specificity as described above. Unfortunately, one of the main mechanisms that tumors employ to evade T cell elimination is downregulation of MHC (137). Chimeric antigen receptor (CAR) bypass this mechanism of immunoevasion by endowing T cells with antibody-specific binding of targets on the cell surface (138). To activate T cells CARs have intracellular signaling domains such as CD3- ζ, CD28, 4-1BB, OX40 among others (129). The correct combination of activating signaling domains is under discussion, but it is clear that addition of second signal activation domains lead to increased persistence and T cell function *in vivo* (139). CAR-T cells have been in development for more than twenty years. Much progress has been made in this time, including the successful application in hematological malignancies.

The treatment of B-cell malignancy in both children and adults with CAR specific for B cell surface markers has proven to be quite effective. The first success with CAR-T was achieved in a patient with B-cell advanced follicular lymphoma who was treated with CD19-CAR-T and underwent dramatic regression (140). This was followed up by the treatment of a CLL patient, where CD19-CAR-T was transplanted at a relatively low dose of $1.5x10^5$ cells per kg but expanded more than 1000-fold. The patient went into remission soon after therapy (141). The second success was in the application of CD19-CAR-T in ALL patients, where potent effects were seen initially in treatment, though eventually one ALL patient escaped the CD19 specificity and progressed (142). The following year an additional study was published where 16 patients with B-ALL were treated with CAR-T. 88% of patients went into complete remission and were moved to standard-of-care allogeneic HSCT (143). In a phase I clinical trial run by the NIH, CD19-CAR-T could be manufactured within 11 days and they reported that all toxicities associated with therapy were reversible, albeit still quite severe (144).

The success of CD19-CAR-T has energized the field of ACT, especially with the adoption of this therapy by pharmaceutical companies such as Novartis, but this success may be limited to therapy for leukemia. One of the benefits of targeting this disease and particularly the antigens CD19 and CD20 is their constant re-presentation in the form of healthy B cells to the CAR-T. Elimination of B cells in

these patients comes with considerable adverse events resulting from humoral immunity, and requires antibody replacement therapy for the foreseeable future for those treated with CD19-CAR-T. Nonetheless, this may be an advantage, as this will ensure long-lived protection from progressing disease. Transferring CD19-CAR into induced pluripotent stem cells provides a proof of concept for generating an unlimited source of CAR-T. These CAR-T were characterized by their similarity in phenotype to γδ T cells and were able to inhibit tumor growth (145). CAR-T have the potential to remodel the tumor microenvironment. CAR-T are able to activate TAM into anti-tumor macrophages that use NO to lyse tumor cells. GM-CSF and IFN-γ produced by CAR-T were found to be responsible for this activation and inhibition of ovarian tumor growth (146). To further facilitate the activation on tumor resident macrophages the CAR-T cells have been modified to express IL-12 under minimal NFAT promoter. IL-12 being present in the tumor led to regression of tumors that were not expressing the CAR specific antigen due to macrophage lysis of tumors (147).

In the treatment of solid tumors CAR therapy success has been less successful. The first trials with solid tumor CAR therapy included targeting carbonic anhydrase IX (CAIX), CD171, FR-α and GD2. For the treatment of renal cell carcinoma with CAIX-CAR-T no clinical benefit was found. Neuroblastoma patients receiving CD171-specific CAR-T had persistence of T cells for 6 weeks but only one patient had a partial response that relapsed once CAR-T were no longer detectable. The treatment of ovarian cancer with FR-α also saw no anti-tumor activity. Furthermore, utilizing HER2-CAR-T containing CD28 and 4-1BB co-stimulatory domains had immediate on-target off-tumor toxicity when transferred into the patient (148,149). This CAR therapy induced cytokine storm that precipitated the death of the patient (150). A more positive result was found for GD2-CAR-T, while initially not showing any direct effect in a long term follow-up study found that low-levels of CAR-T survived up to 192 weeks and mediated a significant survival benefit (151). This strongly suggests that more refined control CAR-T, and a shift of focus away from CAR co-stimulatory domain modification is required for successful solid tumor therapy.

2.1.3.3 NK adoptive cell therapy

While T cells are potent killers when they recognize their cognate antigen, they require specificity to function. As discussed above, it is possible, through complex ex-vivo manipulation, to endow tumor specificity. One of the first clinical trials with adoptive lymphoid cells was done but culturing cells in IL-2, these were termed lymphokine-activated killer (LAK) cells and after transfer in a patient led to a response in 20% of the patients following re-infusion (152). These LAK cells contain a high proportion of NK cells. NK cells, in contrast to T cells, require no antigen specificity and are therefore an interesting modality for adoptive cell therapy. This approach has demonstrated success with hematological malignancies. In other

malignancies it is not yet effective (153). With regard to NK cell function in the context of stem cell transplant, it has been shown that KIR mismatch increases the NK cells activation against their target, leading to improved lysis of leukemia (154). NK cells transferred in patients previously treated with high dose chemotherapy increased engraftment and serum concentrations of IL-15 (155).

2.2 ACTIVE IMMUNOTHERAPY

Inducing an immune response to antigens *in vivo* is the essence of active immunotherapy. The pillars of active cancer immunotherapy include modalities such as DC vaccination, peptide vaccination as well as nucleic acid based vaccination (157).

Edward Jenner laid the foundation for vaccination. The term "vaccine" was coined by his contemporary and fellow vaccinologist Louis Pasteur in tribute to the first vaccine being derived from cowpox. Edward made the observation that milkmaids who had contracted cowpox would not suffer from smallpox. To test this he vaccinated his gardeners' son, James Phipps, by inserting fluid from a cowpox lesion into his skin. The boy had a mild reaction to the vaccination. Six weeks after vaccination Edward took fresh smallpox pustule material and inoculated the boy. Even after repeated challenges James did not present any disease. He late exclaimed: "I hope that some day the practice of producing cowpox in human beings will spread over the world - when that day comes, there will be no more smallpox." The WHO declared smallpox eradicated in 1980.(156)

2.2.1 Dendritic Cell Vaccination

Dendritic cells are at the core vaccination. These cells are responsible for driving immunity against antigens in all vaccine strategies, and since their discovery by Ralph Steinman have been the focus of intense investigation. To date there is one FDA approved dendritic-cell-based vaccination for humans, sipuleucel-T. This vaccine has been approved for use in minimally symptomatic metastatic castration-refractory prostate cancer. Patients treated with sipuleucel-T have a modest increased in median survival of a little more than four months compared to placebo group (158,159). Sipuleucel-T is considered a DC vaccination, but it is a product derived from leukapheresis where granulocytes, lymphocytes and low-density monocytes are removed, leaving behind dendritic-cell precursors. These are then washed and pulsed with a fusion protein consisting of prostatic acid phosphatase (PAP) and GM-CSF. For 40 hours they are incubated in serum/cytokine free medium prior to infusion into patients (160). In contrast to many other clinical DC trials the sipuleucel-T vaccination product does include *ex-vivo* maturation of the DCs (161).

Metastatic melanoma is one of the first tumor types with well-described tumor associated antigens (162,163). This allowed for the development of therapies specifically eliciting responses to these antigens. By pulsing DCs with peptides, Melan-A, gp100, tyrosinase among other melanoma antigens prior to intravenous injection four out of 14 of the patients were able to mount a detectable antigens specific response and two had measurable anti-tumor effects. The DCs were

generated from $CD34^+$ cells, and matured into DCs by culture in cytokine cocktails including IL-4/GM-CSF followed by TNF-α (164). In a similar study, DCs derived from $CD34^+$ progenitor cells pulsed with melanoma antigens generated responses in 16 out of 18 patients. In this setting immunogenic antigens, KLH and Flu peptides, were pulsed along with melanoma antigens on the DCs. The two patients who did not react to melanoma antigens or control antigens had rapid progression of disease, while all other patients had an immunological response to at least one of the melanoma antigens. In this study overall immunity to melanoma antigens was associated with improved clinical outcome (165). A target for immunotherapy that remains intriguing is the unique tumor antigen expressed by B cell lymphomas (166). B cell lymphomas can be targeted based on their idiotype (Id), the unique variable regions of the clonal immunoglobulin. In the case of B-cell lymphoma this is expressed on all cells of the disease, as it is clonal in nature. DCs were generated from PMBC purified by leukapheresis and pulsed with Id-purified proteins together with an immunogenic carrier protein KLH. DCs were reinfused into patients and were able to generate cellular or humoral responses in 65% of patients. Six patients who did not have direct benefit were boosted with Id-KLH-DC vaccines, of which two had complete response and one had a partial response (167). Proceeding with leukapheresis to procure PBMC from which generate DCs, particularly when harvesting monocytes from which to generate DCs, in cancer patients comes with the inherent issue of co-purifying monocytic MDSC that inhibit maturation of DCs (168). Additionally, GM-CSF used to mobilize $CD34^+$ cells from the bone marrow is able to recruit MDSC(169). To overcome the recruitment and isolation of MDSC, but also to initiate a type I interferon centric type of immune response, pDCs have been used to vaccinate metastatic melanoma patients. Via apheresis pDC were directly isolated from patients using magnetic bead sorting based on pDC cell surface marker BDCA4. Melanoma antigens were pulsed onto DCs in combination with FSME-IMMUN for activation of pDC. The patients were injected subcutaneously and pDCs were able to migrate to multiple lymphnodes. These patients had a overall survival of 22 months which was significantly improved when compared to historical controls that had an overall survival of 7.6 months (170).

With innumerable DC therapies ongoing, and 43 clinical trials being completed last year alone, interest in using this modality of immunotherapy remains strong (161).

2.2.2 Peptide Vaccination

Peptide vaccinations rely upon the knowledge which antigens are expressed by the tumors. All different classes of tumor-associated antigens have been targets of vaccination with the most impressive results being generated in peptide or protein vaccine approaches using non-self tumor specific epitopes. Vaccination against non-self peptides evades the central tolerance trimming of potential anti-tumor specific T cells. HPV16-induced gynecological carcinomas are a prime target for this mode of

vaccination. These develop from the premalignant states that are caused by high-risk types of HPV. When patients in this premalignant state are vaccinated with overlapping 25-35-mer peptides covering HPV16 E6 and E7 proteins 9 out of 19 patients had complete remissions of their disease (171). When long-peptide vaccination was used in advanced gynecological carcinomas, T cell responses were elicited, but these were restrained by tumor mediated immune suppressions so while it was possible to elicit a response no direct clinical effect was noted (172). Melanoma, and melanoma-associated antigens have also been used for peptide-based vaccination. TAA gp100 has been used as a target of peptide vaccination. In a clinical trial commenced after the discovery of this TAA, two 9-mer peptides were mixed with IFA and patients were vaccinated thrice in combination with high dose IL-2. Surprisingly, 42% of 31 patients had objective responses (173). In a follow-up phase-III trial, comparing IL-2 with vaccination using gp100 peptides combined with IL-2, a less stellar response was noted with only 16% response rate. This was still significantly higher than IL-2 alone which had a response of 6% with 11.1 months overall survival compared to 17.8 with peptide vaccination (174). In RCC, another immunogenic tumor, vaccination with a multi-peptide vaccine IMA901 which contains epitopes for antigens such as Cyclin D1 and MUC1 was able to generate responses to multiple epitopes (175). This vaccine, in combination with cyclophosphamide, elicited immune responses that were associated with clinical benefit, particularly in patients with low suppressive cell populations (176).

While peptide vaccines get to the heart of vaccination they must overcome hurdles that other modes of vaccine do not need to overcome. As DCs remain the lodestone of vaccine strategies, injection with peptide alone would generate a weak or possibly toleragenic response, hence the requirement of adjuvants to generate “danger signals” in DCs. These include adjuvants such as montanide, incomplete Freund’s adjuvant, monophosphoryl lipid A, BCG as well as imiquimod that are able to activate PRRs through various mechanisms and activate DCs (177). Additionally, fusion protein-peptides that contain DC ligands have been developed to ensure that the peptide is presented by APCs which is optimally effective to deliver antigen (178). A MUC1 fusion protein based vaccine approach targeting mannose receptor in the setting of stage II breast cancer has shown remarkable effect. Out of 16 patients vaccinated only two had a recurrence, while in the placebo group the expected recurrence rate of 60% in 15 patients was noted (179). Further augmenting epitope responses can be achieved through modification of single or multiple amino acids in the peptides used for vaccination. This was indeed already the case for the gp100 vaccinations where a threonine was replaced with a methionine led to increase overall responses in patients (173). Modification of other melanoma antigens has led to increased binding to MHC and directly led to increased levels of IFN-γ production in T cells and increased anti-tumor immune response (180).

2.2.3 Nucleic Acid Vaccination

With nucleic acid vaccination we take an extra step further from directly inducing immunity by DCs primed ex vivo and peptides loaded onto DCs in vivo to genetic information encoding TAAs. With the advent of molecular biology, the manipulation of nucleic acids to express the desired antigens, fusion proteins, cytokines or other immunostimulatory proteins becomes accessible and feasible. In addition, to being especially suited to manipulation, nucleic acids are innately immunogenic, as they are natural “danger signals”. While peptide and protein vaccines rely on adjuvants, nucleic acids are their own adjuvant and are able to bind to the many DAMP binding PRRs described above (181,182).

The delivery of nucleic acids for vaccination can be done using various vectors, including viral, bacterial, naked plasmid DNA or naked RNA. Initial findings for the use of DNA vaccines against viral antigens was that the therapy was well-tolerated, with no integration of DNA into the genome, no autoimmunity and no tolerance, and was able to induce both cellular as well as humoral immunity in patients (183). DNA vaccines have generally been delivered either intramuscularly or intradermally. In both cases antigens are presented in either a direct or indirect route. Antigen encoding DNA delivered directly into DC cells allows for direct presentation of TAA epitopes. APCs can also collect antigens through cross-presentation facilitated by the uptake of DNA by keratinocytes or myocytes. Thus DCs are able to elicit both $CD4^+$ as well as $CD8^+$ T cell responses (184).

The first attempt at utilizing DNA vaccines was targeting immunogenic antigens such as those found in influenza. Mice vaccinated with nucleoprotein encoding DNA mounted cytotoxic $CD8^+$ T cell as well as antibody responses (185). This provided the basis of expanding DNA vaccination to tumors. The first DNA vaccine targeting human tumors was in B-cell lymphomas targeting the Id region. Hawkins *et al.* were able to show that it was possible to easily generate patient-specific DNA vaccines that would produce antigen that would be recognized by the patients’ immune system (186). Using this technology a phase I study was set up to test the safety and feasibility of DNA vaccines targeting Id. The Id was fused to mouse Ig constant regions in a plasmid DNA vaccine delivered using a gene-gun. Six out of 12 patients were able to mount humoral or cellular anti-Id responses though no clinical response was observed (167). Other hematological malignancies could be targeted through vaccination against WT1 tumor antigen using rationally designed DNA vaccines. In HHD mice, highly potent CTLs were generated that were able to kill WT1 expressing tumor cell lines (187). As with peptide vaccines, DNA vaccines have been utilized to generate responses against melanoma antigens. In a study to compare xenogeneic responses, 18 stage III and IV melanoma patients were randomized into groups where either three vaccines with mouse tyrosinase was followed by three vaccines

with human tyrosinase or vice versa. Xenogeneic vaccination had no impact, but overall seven out of 18 patients generated T cell responses to native tyrosinase peptide. When, compared to historical controls, patients on trial had a median survival of 42 months compared to 36 months (188). DNA vaccination using plasmid DNA encoding HER2/neu in combination with IL-2 and GM-CSF as adjuvants, was able to generate both humoral and cellular immunity in metastatic breast cancer patients, though once again no clinical benefit could be demonstrated (189).

Some successes have been recently made with DNA vaccines. A DNA vaccine approach targeting MAM-A, a TAA expressed by 40-80% of primary breast cancers, was delivered IM with a Needle Free Biojector and patients were able to generate de novo responses to this MAM-A. Vaccination was able to induce $CD8^+$ T cell antigen specific IFN-γ response in eight out of eight HLA-A2 patients. Impressively, at six months 53% of patients were progression-free while 33% of the patients not meeting the HLA-based inclusion criteria for the vaccine remained progression free (190). DNA vaccines in stage III HPV-positive cervical intraepithelial neoplasia patients were able to elicit $CD8^+$ T cell responses against E6/E7 that resulted in complete clinical regression of lesions and viral clearance. DNA vaccines were delivered via intramuscular electroporation generating potent multifunctional T cell responses (191). DNA vaccination has also been tested in prostate cancer, eliciting CTL and antibody response has been possible but was not effective clinically (192,193). Combining immune-checkpoint blockade antibodies together with vaccination is able to release anti-tumor vaccine generated immunity. This has been shown to be the case for PD-1 and PD-L1 checkpoint blockade in combination with prostate cancer antigen DNA vaccine SSX2 (194).

A platform of nucleic acid based vaccination that is resurging to prominence is the delivery of in vitro transcribed (IVT) RNA. After an extended hiatus, due in part to perceived instability, RNA vaccines are showing some unique advantages over DNA-based vaccination. Namely that IVT RNA does not need to enter the nucleus to be functional, and is immediately translated upon cellular uptake. IVT RNA based vaccines are also transiently active and metabolically degraded in a short amount of time (181,195). In addition, very low doses of RNA, in combination with α-virus replicon technology, are able to generate virus specific antibodies and T cells. This would allow for improved scale up from rodents to humans (196). The ease of manipulation of nucleic acids allowed for the generation of Id patient specific DNA vaccines, and now RNA vaccines are being utilized for the generation of neo-antigen based vaccines. This is a clinical trial to keep an eye on for the potential therapeutic application of future cancer vaccines (197).

3 AIMS OF THE THESIS

The general aim of this thesis is to progress the fields of both passive and active cancer immunotherapy. In recent years both have been making great strides in therapeutic outcome for patients. While progress is encouraging, many obstacles have not been properly addressed. In this thesis I address the hurdles of tumor mediated immune suppression in passive immunotherapy as well as furthering the understanding and boosting active immunotherapeutic reaction against known as well as novel tumor antigens.

Strengthening the pillar of passive immunotherapy

Paper I. Catalase protects tumor redirected T cells from oxidative stress. Tumor cells and associated suppressive cells have the capacity to generate abnormally large quantities of reactive oxygen species that lead to oxidative stress and immune dysfunction. We modified T cells *ex-vivo* with chimeric antigen receptors, to endow them with tumor specificity, as well as with catalase to offer protection from oxidative stress that allows modified T cells to remain functional under oxidative stress.

Strengthening the pillar of active immunotherapy

Paper II. Intradermal DNA vaccine induced anti-tumor immunity requires NF-κB. Vaccination with antigen encoding DNA is able to elicit a specific cytotoxic T cell response. The mechanism with which this immunity is established was explored in multiple knockout mouse models. We were able to identify that some expected pathways were not players, and the majority of anti-tumor activity rested on NF-κB activation.

Paper III. Delivery of a genetic adjuvant boosts anti-tumor immunity. Based on the previous findings, we strove to increase anti-tumor CTL responses to antigen encoded by the DNA through the addition of plasmid DNA encoding a genetic adjuvant. This led to a type I IFN dependent increase in tumor specific CTLs that were able to increase survival in tumor challenged mice.

Paper IV. Protection against metastatic cancers could be induced by vaccination targeting Cripto-1. The success of cancer vaccines rests on the antigens that are in the arsenal of immunotherapists to utilize for vaccination. We developed a cancer vaccine that is able to elicit adaptive immune responses against Cripto-1 leading to decreases in metastatic spread as well as inhibiting the growth of cancer stem cells.

4 RESULTS AND DISCUSSION

4.1 PROTECTING TUMOR REDIRECTED CAR-T CELLS FROM ROS

Co-expressed catalase protects chimeric antigen receptor-redirected T cells as well as bystander cells from oxidative stress-induced loss of anti-tumor activity

Manuscript

Tumor infiltrating lymphocytes are associated with better prognosis in a variety of cancer types. Therapy using TIL expanded *ex-vivo* has been established as a remarkably effective passive-immunotherapy approach for metastatic melanoma patients. This highlights the potent nature of adoptively transferring lymphocytes, but unfortunately it is not always possible to generate TIL for each patient, particularly TIL that are reactive to their malignant clone. Modification of T cells with CAR *ex-vivo*, a trans-membrane fusion protein that endows T cells with tumor specificity as well as activation potential, is an alternative to enriching tumor reactive T cells from TIL. CAR modified T cells have been successfully applied to acute as well as chronic lymphocytic leukemia, and may soon become a replacement for standard therapy for this disease. Treating solid tumors with CAR-modified T cells has proved to be less effective. It is not only required of the T cells that they recognize and target the tumor; they must also be able to survive and retain their function in the tumor microenvironment. One of the factors leading to immune-dysfunction in the tumor microenvironment is the high level of oxidative stress that are encountered by the transferred lymphocytes. ROS production is not exclusively the domain of the tumor cells but also myeloid derived suppressor cells, and regulatory T cells as well as granulocytes recruited by the tumor. In this study we attempted to relieve the burden of oxidative stress by endowing CAR redirected T cells with catalase.

We confirmed that the retroviral CAR and catalase encoding bi-cistronic expression vector transduced T cells and lead to significant increases in catalase activity. T cells transduced with CAR and catalase (CAR-CAT) were found to have lower basal oxidative stress than T cells transduced with only CAR. CAR-CAT T cells were able to maintain a reduced state during activation with mitogens while CAR T cells became stressed. The stress induced by H_2O_2 led to dysfunction in CAR T cells but not in CAR-CAT T cells. This was found to be the case for both proliferation as well as specific cytotoxic tumor lysis by the T cells. In addition to a direct benefit to the CAR-CAT T cells, we found the these transduced T cells were able to mediate a beneficial bystander effect; leading to reduced bystander lymphocytes which were able to maintain lower oxidative state as well as preventing loss of CD3-ζ. In the case of bystander NK cells, CAR-CAT T cells were able to maintain NK cell cytolytic function.

The tumor microenvironment is a complex mix of cells, signaling and of constant flux. It is in this environment that CAR transduced T cells must enter and commence their elimination of the tumor mass. Researchers have long been struggling to optimize the CAR fusion protein directly. The discussion between whether CD28, 4-1BB, OX40 or other co-stimulatory fusion combinations has dominated the landscape of CAR modified T cells. With this work we attempt to steer the conversation on adoptive T cell therapy in the direction of addressing the other obstacles present in the tumor microenvironment.

In the tumor microenvironment, one of the hurdles that needed to be addressed is oxidative stress. The CARs used in this study were directed at CEA and HER2, both tumor antigens expressed commonly on a variety of breast cancer and colorectal cancer. Both these tumors are typified by their high ROS production and associated oxidative stress; lymphocytes present in both diseases have been found to have high levels of oxidative stress. We revealed that by co-expressing catalase in T cells together with CARs led to reduced lymphocytic oxidative stress and maintained T cell functionality. In the case of adoptive cell transfer, there is a requirement for rapid expansion of cells *ex-vivo*, typically leading to the generation of effector and effector memory T cells, both subsets of T cells more susceptible to oxidative stress than their naïve T cell counterparts. We also found that non-transduced bystander cells were protected from oxidative stress when co-cultured with CAR-CAT transduced T cells. In the tumor microenvironment ROS has an extremely detrimental effect on NK cells, and may be one of the largest factors to their nearly non-essential role in most tumors. Combining tumor redirected CAR T cells with transgenic expression of molecules that lead to resistance of adoptively transferred cells to the suppressive tumor microenvironment may bring about the success of this therapy beyond the scope of its use now.

4.2 NF-ΚB LINKS DERMAL DNA VACCINATION TO IMMUNITY

NF-κB activation during intradermal DNA vaccination is essential for eliciting tumor protective antigen-specific CTL responses

Human Vaccines & Immunotherapeutics 2013, 9:10, 2189-2195

Activating a tumor specific cellular adaptive immune response has been a long-standing goal in the field of cancer-vaccines. DNA vaccines provide an interesting platform for the discovery as well as delivery of novel tumor associated antigen based vaccines (198). Delivery of DNA vaccines has found two main sites of interest, namely intramuscular and intradermal, and with the assistance of electroporation leading to increased CTL responses to the encoded antigens (199,200). Delivery of DNA to the dermis is of particular interest due to resident APC, both Langerhans cells as well as dermal DCs, present at this site. These cells are proficient at processing the antigen as well as migrating to lymphoid organs and eliciting antigen specific responses (201). The activation of resting DCs is mediated through potentially a plethora of pathogen-recognition receptor. DNA found in the cytosol, such as after electroporation based DNA vaccine delivery, is a potent danger associated molecular pattern leading to the triggering of NF-κB as well as IRFs. Improving the efficacy of DNA vaccines requires a more thorough understanding of the pathways and transcription factors involved in eliciting a protective anti-tumor immune response.

DNA vaccines encoding antigens, either highly immunogenic model antigen ovalbumin (OVA) or tumor associated antigen tyrosinase related antigen 2 (TRP2), were delivered using intradermal electroporation. Cytotoxic T lymphocyte responses were evaluated using intracellular cytokine staining as well as *in vivo* cytotoxicity assays. Type I interferons are one of the key cytokines responsible for linking innate and adaptive immunity; this link is laid through the common IFN-α receptor as well as through the activation of transcription factor IRF3. IFN-α receptor and IRF3 knockout mice showed no decrease in peripheral blood activated CTLs, nor was the function of CTLs impaired in IFN-α receptor knockout mice. We evaluated the role of classical bacterial DNA sensor TLR9 and its adaptor protein MyD88 to mediate a role in establishing CTL based immunity, but found neither to play a role in either eliciting CTLs or dampening their function. Inflammatory cytokines IL-1 and IL-18 have been described to be regulated in part by AIM2, a cytosolic DNA sensor that activates caspase-1. In IL-1/IL-18 KO mice vaccination was not found to decrease CTL induction or efficacy compared to WT mice. NF-κB, a potent regulator of cell survival and inflammation, on the other hand was found to be activated by DNA vaccination and to be responsible for mediating TRP2 specific CTL responses. Most importantly, the decreased responses by inhibition of NF-κB signaling abrogated the anti-tumor response generated by DNA vaccination.

While it is not wholly surprising that NF-κB can play such an essential role in eliciting immunity, our findings that type I interferon were not required was surprising. The field of DNA-sensing innate immune receptors has expanded enormously in the past few years, including many new molecules and pathways, but essentially leading still to either the activation of IRFs or NF-κB. Previous studies have shown that in TBK-1, IRF3 and IFN-α receptor pathway knockout mice DNA vaccine elicited CTL responses were almost completely eliminated (202,203). These findings are in stark contrast to the results generated in our model, and yet can be reconciled due to the fact that delivery of DNA in those studies was done intramuscularly. It is not unreasonable to consider that the location of vaccine delivery will have an impact on antigen immune responses. Indeed, α-virus replicon-based vectors, which lead to the production of large amounts of cytosolic RNA, were hampered by type I IFNs when delivered intradermally (204). Activation of NF-κB in DCs leads to the production of potent inflammatory cytokines, including IL-12, IL-6 and TNF-α, up-regulating secondary signaling, including CD80 and CD86, as well as increasing survival, by regulating BCL-2, Bcl-XL among others (205). Uncovering the role of these transcription factors opens up the field to direct improvement of DNA vaccines.

4.3 DAI BOOSTS DNA VACCINE BASED ANTI-TUMORAL IMMUNITY

DAI (DKN-1/ZBP1) as a Genetic Adjuvant for DNA Vaccines that promotes effective antitumor CTL immunity

Molecular Therapy 2011, 19:3, 594-601

DNA vaccines, while an attractive choice for antigen delivery due to its safety and ease of production, have yet to have the same impact in the clinic as has been shown in animal models (182). Tumor specific CTLs are implicated in favorable clinical prognosis of cancer patients. Eliciting a CTL response potent enough remains challenging for DNA vaccination; there are many underlying issues for these frail immune responses, one of which is that tumor-associated antigen epitopes detectable by T cells are normal non-mutated self-antigens that are either negatively selected against or become T regulatory cells. To ameliorate these issues proper delivery of DNA vaccines and strong adjuvants are necessary to generate a potent anti-tumor immunity. Clinical trials comparing electroporation of DNA vaccines to standard delivery have been able to show that this method of delivery to be much more superior than without electroporation. The co-delivery of cytokines, and even co-delivery of cytokine encoding DNA, indicate the importance of generating the correct immune context to induce potent CTLs (206). In this study we look upstream of these cytokines and endeavor to harness a cytosolic DNA sensor, DAI, to engage maturation of dermal DCs and activation of T cells through its co-delivery with an antigen encoding DNA vaccine.

DNA vaccine delivery via intradermal electroporation has been shown to induce the transcription of many pro-inflammatory genes. We found that in addition to these already increased levels, co-administration with DAI encoding plasmid DNA (pDAI) significantly increased type I IFN gene expression as well as markers related to DC maturation and T cell activation. Co-delivery of pDAI with antigen-encoding plasmid DNA stimulated increased *in-vivo* proliferation, antigen specific cytotoxicity and *ex-vivo* antigen specific cytokine production. Tumor associated antigen vaccination in combination with pDAI enhanced anti-tumor responses significantly, endowing challenged mice with increased survival. Mice that survived the primary challenge were re-challenged and found to be protected by a memory response that was not present in TAA-DNA vaccinated mice alone. Evaluation of antigen specific T cells revealed that using pDAI in combination with antigen encoding DNA for vaccination significantly increased the percent of effector memory ($CD44^{high}$ $CD62L^{low}$) and central memory cells ($CD44^{high}$ $CD62L^{high}$) T cells; this corresponded nicely with increased survival after tumor re-challenge. Additionally, we evaluated the mechanisms involved with the adjuvanting effect of pDAI co-delivery and found them to be mediated by both NF-κB as well as type I IFNs.

In our previous study we found that type I IFNs were dispensable to tumor specific CTL responses elicited by DNA vaccines. We speculated that by introducing an intracellular PRR as a genetic adjuvant we would be able to harness a wide variety of proinflammatory signaling molecules activated by PRRs to enhance the DNA vaccine induced response. It is important to note that previous studies have employed encoding cytokines on plasmid DNA to adjuvant DNA vaccine responses (207,208). These have shown increased anti-tumor effect, on the other hand, these strategies may lead to the recruitment of suppressive T regulatory as well as myeloid suppressor cells (209,210). While employing an upstream regulator of a plethora of inflammatory responses we can abrogate the recruitment of suppressive cells and induce a potent anti-tumor immunity.

4.4 ONCOFETAL PROTEIN CRIPTO-1 IS TARGETABLE BY DNA VACCINATION

Vaccination against tumor-associated antigen Cripto-1 elicits a protective immune response to metastatic melanoma and breast cancer stem cells

Manuscript

Vaccination against pathogens has protected the lives of billions of people. Using this treatment modality to treat cancer patients has left much to be desired, as most responses generated in patients have been underwhelming at best. Though immunologists should remain steadfast in their resolve to generate vaccine induced responses potent enough to mediate protection in patients. In the previous work we have demonstrated a method to enhance CTL responses, but development on targeting novel antigens is required as well. To date many tumor associated antigens, such as MAGE-A, gp100, Ny-ESO-1, Tyrosinase and HER2 to name a few, have been targeted by vaccination but have been met with limited success (211). It may very well be that these antigens, while up-regulated on bulk tumor, were not the optimal targets for the application of cancer vaccines. Cripto-1 (CR), a small cell surface glycoprotein, is an interesting target in that it has been shown to be involved in rapid proliferation, angiogenesis as well as epithelial-mesenchymal transition (212). Additionally, breast cancer patients with high levels staining for CR have poor prognosis (213). Interestingly, CR has been identified on cancer stem cells as well (214). These are small populations within the tumor mass responsible for generating the daughter cells that drive tumor growth. Additionally, cancer stem cells are resistant to classical cancer therapies, including radiation, chemo and surgical therapy. These attributes constitute the hallmarks of progressive disease and targeting these pathways with vaccination may have a greater impact than targeting general tumor-associated antigens.

Mice were vaccinated using plasmid DNA encoding for mouse CR (mCR). Prophylactic vaccination in C57BL/6 mice reduced B16F10 transplantation growth as well as increasing survival of vaccinated mice. More impressive results were obtained when vaccinated mice were challenged intravenously, significantly reducing lung metastasis. We identified three potential epitopes by stabilizing MHC class I molecules on RMA-s cells. Two of the epitopes were able to stimulate *ex-vivo* peripheral blood lymphocytes to produce IFN-γ and TNF-α. $CD8^+$ T cells isolated from vaccinated mice produced IFN-γ upon co-culture with B16F10 as well as stimulation with mCR_{16-25}. To explore whether mCR vaccination would be effective in additional models, BALB/c mice were vaccinated and we found that they were able to produce antibodies specific for mCR. When challenged with a spontaneously metastasizing breast cancer model the vaccination was able to very significantly reduce the metastatic index by 6 fold, while the effect on tumor mass was a significant reduction of 1.5 fold. To evaluate the effect of vaccination on cancer stem

cells (CSCs), a tumor cell line expressing low levels of mCR was cultured in mammospheres. Each progressive passage on low attachment plates increased mCR expression allowing vaccination against the target to decrease tumor growth as well as improving survival. In a therapeutic setting, BALB-neuT mice were vaccinated in week 10, when female mice typically already have developed *in situ* carcinoma. Vaccination in this setting significantly reduced metastatic burden.

In this study we show that vaccination against CR induces a cellular and humoral responses in a variety of mouse models. These responses are able to inhibit tumor growth, though this was not found to be the case in the BALB-neuT mice. While the vaccination was not able to restrain solid tumor growth, it was able to have a very large impact on the spread of metastatic disease. In addition to halting metastatic spread, vaccination of mice against CR endowed mice with anti-tumor immunity that was able to hinder the growth of transplanted cancer stem cells. CSCs have been shown to have increased expression of CR in melanoma, prostate and breast cancer (215-217). CSCs in breast cancer have high metastatic potential. This highlights the efficacy of anti-tumor immunity against small groups of cells that are essential to eliminate to increase survival of cancer patients.

5 FUTURE PILLARS OF CANCER IMMUNOTHERAPY

With growing success of passive immunotherapies, and the increasingly positive results from active immunotherapy trials, there is a renaissance occurring in cancer immunotherapy that is translating to novel treatments that, in contrast to many classical therapies, are able to cure cancer patients. But, of course we are in the field of cancer research, and while not all cancer patients are treatable we must pursue improvements. There are innumerable ways forward, but I will highlight a couple that I find promising.

5.1 HARNESSING THE POTENCY OF ADOPTIVE CELL TRANSFER

Adoptive cell therapy, particularly in the form of CAR redirected cell therapy, is coming into the main stream. With companies such as Novartis investing in large-scale production facilities (buying out Dendreon), we should soon see CD19-CAR-T treatment for the masses. Of course, harnessing the potent cytolytic potential of T cells has worked well for hematological malignancies; it is yet to be similarly successful in solid tumors.

Modifying T cells through the use of viral vectors and transposons allow for further modification beyond delivery of CAR. In our study we transferred catalase to offer protection from the suppressive tumor microenvironment. This concept is being pushed forward in our lab at the moment with delivery of TRX1 and silencing of KEAP1 to further facilitate protection from oxidative stress. This is of course just one aspect of immunosuppression that can be targeted; already a double-negative receptor for TGF-β has been shown to covey resistance to T cells from this suppressive factor (218). Targeting checkpoint inhibitory molecules is a prime target for improvement of ACT, especially in light of clinical success of these antibodies. To achieve this various new techniques, such as CRISPR/Cas, ZFN or RNAi, can be utilized. Recently, this has been achieved in TIL from melanoma patients in which ZFN targeting PDCD-1 gene knocked out 74.8% of the PD-1 encoding gene. This led to increased proliferation and enhanced polyfunctional T cells (219).

Expression of IL-12 in a NFAT regulated manner in both CAR as well as TIL therapy modalities brings potential to remodeling of the tumor microenvironment. In a recent clinical trial using TIL with IL-12 production 63% of patients had objective response at a TIL transfer rate 10-100 fold less than in typical TIL therapy. Serious adverse events clearly indicate that an approach like this requires acute control and monitoring (220). In mouse models for CEA, CEA-TRUCK-T (fourth generation CAR(147)) have proven to mobilize and re-educate M2 TAM into anti-tumor macrophages. This enables the elimination of TAA negative tumors along with positive tumors (221).

Finally, adoptive T cell transfer is a modality that has shown to be potentially fatal. It is important to harness these potent cytolytic cells, and it should be reasonable to

consider building in kill switches (222) or utilize RNA electroporation of T cells. This would minimize on-target but off-tumor adverse events.

5.2 EXPLOITING WINDOWS OF OPPORTUNITY

The official acceptance of vaccination, particularly the approval of sipuleucel-T as a therapy for prostate cancer by the FDA, is a boon to field of cancer vaccines. It shows that there is certainly room for vaccination in our field. The demise of the company producing the sipuleucel-T vaccine also highlights the enormous hurdles needed to be overcome by cancer vaccines. Sipuleucel-T vaccine induces a measurable immune response in most patients, but again, this immune response was only able to show a very limited increase in survival. In our work we have tried to address some of these issues, but there is much more room for strengthening this pillar of cancer immune therapy.

The first, most obvious room for improvement is finding the window of opportunity for vaccination, particularly when considering that ten vaccines should be given against hepatitis B, rotavirus, diphtheria, influenza and many others before children have become one year of age. Additional vaccines and booster shoots are given up to 18 years, as recommended by the CDC. Of interest is the HPV vaccine, which is recommended to be initiated at a range of 11-12 years old though may be taken in females from 13-26 (223). The goal of HPV vaccination is to prevent the development of virus induced cancers. Vaccination at this stage in the life cycle of humans is due to the ability of younger children to develop potentially life long immunity against a large variety of diseases. At the moment, therapeutic vaccination is the only option available for inducing immunity against cancer. Cancer is often considered a disease of the elderly, there are of course tragic exceptions, but nonetheless 36% of all cancer cases are diagnosed in people above 75 years of age. Age comes with natural immunosenescence. The nature of immunosenescence is such that innate immunity is compromised; HSCs are less active and have a skewed preference towards generating myeloid cells (224), a marked reduction in the ability of neutrophils and DCs to phagocytose (225). Immunosenescence leads to changes in adaptive immune responses as well; T-cell diversity is compromised with expansions being only clonal in nature (226). Dysfunction in innate immunity leads to dysfunction in adaptive immunity (225).

These issues are compounded when taken in the context of cancer patients, where the tumor induces a systemic immune suppression. In the context of vaccination, MDSC have found to be inversely correlated to response (227). In addition to the prognostic value of MDSC to response in RCC patients, the burden of Tregs was an additional prognostic marker. Depletion of Tregs by cyclophosphamide increased overall survival in patients (176). Taken together, this makes a strong argument for the window of opportunity being when the prospective cancer patient is treated with vaccination at a younger age. A truly prophylactic anti-cancer vaccine would be able

to enhance immune surveillance and reduce cancer development. A promising direction is being taken in the vaccination against MUC1 in patients with pre-CRC lesions (228). Cervical cancer is a cancer that may also be an appropriate vaccine candidate in this context, it can have visible pre-malignant stages which allow for vaccination to be applied in a relatively healthy patient (229). Pre-malignant cervical cancers have a 30% chance of becoming invasive cancers (230). A DNA vaccine has recently shown promising results (190), building upon the success of long-peptide vaccines (171). Additionally for other cancers such as malignant melanoma, metastatic breast cancer and colorectal carcinoma pre-malignant stages are frequently identified and would provide many opportunities for inducing immunity in non-suppressed, younger patients (231).

When therapeutic vaccination becomes a necessity, combination therapy must be used to force a window of opportunity. Potential for combinations is nearly endless, and many arguments could be made for all. Briefly, surgery has been shown to reduce MDSC in breast cancer models (232) as well as CRC patients, where Tregs were additionally reduced (233). Targeting of Tregs can be done with anti-CD25 to augment peptide based vaccination (234). In combination with chemotherapy, peptide based vaccine strategies synergize to produce robust TNF-α and IFN-γ leading to reduction of MDSC and increased survival (235). Cyclophosphamide has been shown to reduce Treg levels and increase effectiveness of vaccines (176), but may also have a detrimental effect on anti-tumor immune compartments by recruiting MDSC (236). Exposure of tumors to ionizing irradiation reduces MDSC and changes suppressive immune infiltrate to an immunogenic one (237). Additionally targeting MDSC can be done in a novel manner by targeting their metabolism by inhibiting fatty acid oxidation. The delivery of etomoxir, an inhibitory of fatty acid oxidation, allowed for increased efficacy of vaccination against tumors (238). TAMs are potentially another cell population that either needs to be reprogrammed or eliminated. This can be done by targeting CSF-1R (239) or through the vaccination response itself. Vaccine induced T cells have a direct effect on being able to shape macrophages from an M2 to M1 phenotype, and these interactions and changes of the tumor microenvironment are essential to the success of peptide based vaccination (240).

Finally, antigens are a major consideration for vaccination and which to target is central to each application. Neoantigens pose an interesting opportunity for vaccination in a therapeutic setting as theoretically they are novel and central or peripheral tolerance will not hinder their immunogenicity. Many trials are ongoing, both as vaccines but also for adoptive cell therapies, attempting to harness these antigens. But while this therapy may not suffer from tolerance, it will suffer economic hardships as the feasibility of generating neoantigen therapies are beyond the means of many. Driver mutations provide interesting alternatives to patient specific neoantigen vaccines or adoptive cell therapy. Such a mutant has been described in

the protein IDH1 that is expressed by a large majority of grade II or III gliomas. Vaccination with long peptides in this case is applicable to a wide variety of patients while still having the advantage of being a neoantigen (241,242). Additionally, while neoantigens may provide the foundations of a new pillar of cancer immunotherapy, it is key to consider that recent findings have once again highlighted that central tolerance may not be as eager to eliminate self-reactive T cells as previously expected (243).

The pillars of cancer immunotherapy are being built upon a solid foundation of oncology and immunology provided by decades of dedicated researchers. Many aspects of these cancer immunotherapies will continue to evolve in the coming decades, pushing the boundaries of which diseases are treatable and how well the patients will respond to immunotherapeutic treatments. I am looking forward to future where we can vaccinate and transfer anti-tumor immunity to protect and cure cancer patients. I hope that my contribution of mortar, stones and rubble have helped solidify and strengthen the pillars of cancer immunotherapy.

6 ACKNOWLEDGEMENTS

I feel like I spent far too much time here in Sweden, but in the same sentence I feel I should express that I would love to spend even more time here at the first floor of the CCK. This dichotomy can exist due to the fact that my life has been split between my fantastic work environment in Sweden and my fiancée (if I'm lucky this coming week) back in the Netherlands.

To my supervisors:

Rolf, you have been an incredible supervisor. I think it is absolutely unbelievable that you have managed to push and pull me through these last few months of writing. It turns out that I am not an immovable object. The time I have spent under your tutelage have shaped me into the researcher I hoped to become. It is unbelievably rare to find a supervisor that allows their students sometimes entirely too much freedom to develop and pursue their own ideas, theories and fantastical imaginations. Thank you for this experience and time, I am forever grateful.

Alvaro, your fuego is what lit my passion for cancer immunology. When I first came to Sweden you took me under your wing and showed me the ropes. Together we had some great discussions while electroporating the rear ends of mice; this type of discussion is how I now strive to teach my students as well, it really brings out the best in us.

Dimi, you have been a real inspiration to me. This is due in part because I am sometimes in awe of how crazy dedicated you are. You manage to be a full time doctor as well as a crazy great scientist at the same time. Your critical comments have pulled the best out of our work together, and keep me striving for better in all my experiments.

Andreas, I am very glad that you have been able to share just a couple of your NIH stories with me. It is a pity that I won't be staying longer to hear more of your adventure there. I kid, its been great to be able to walk in and get your advise on all things including discussing my ongoing projects as well as our now ongoing seafaring (or not) adventures.

Roland, as my external mentor I would like to thank you for spending a couple afternoons talking science and for connecting me to others in the field. I look forward to seeing you again and I hope that we can continue this mentorship after my time here in Sweden.

To my lab-mates:

Here on the first floor there is a tight knit community of researchers whom together work tirelessly at bringing about the realization of strong pillars of cancer

immunotherapy. I could never have done this work without their contributions, comments and constructive criticisms.

Mao, together we started our PhD studies, with you just being a couple months older, both in study time and real life. I have always had an older brother, someone to watch as well as ask when things got a little tricky, you have been my PhD brother these last four years. It's been a real pleasure going through this process together with you. I don't need to wish you much luck with your research; I'm sure you'll blow everyone away at your next position. But I will wish you best of luck under the thumb your Nina. **Kristina**, I can't believe how lucky I was to get a student like you. When you first arrived, you were a little uncertain, but soon you grew into a fantastic researcher and friend. Together we made easy work of the Cripto project while dancing to music in the middle of the night in the little lab. Keep that attitude and you'll do great. And good luck with the flights, its tough. **Yago**, you have been a good influence on me, sheading light on the "self-enrichment predicament" and how we must try to find a balance. I hope you and Patricia land on your feet well back home in Spain soon. **Tanja**, we have had some great times drinking coffee and making "the appropriate" jokes. **Jeroen**, you've been at the lab for less than two years yet I feel like you've been here forever, thanks for the questions and making the lab meetings interesting. **Yuya**, although you left recently back to your work as a Radiation Oncologist in Japan I will make sure that our work will continue and I look forward to seeing you at a conference soon. **Maria and Ulrika**, thanks for being here and helping with all the things, without you guys that lab would fall into disarray. **Lars**, thanks for the help with the reagents and for the great hunting stories, I hope you catch a big moose next season! **Iva**, I am very happy that you will be joining our lab next spring, you've joined the group and fit like a glove. **Bettina**, it has been great having you around, I wish we could have spent more time playing board games though, maybe I'll have to visit Bavaria next winter for a game of Roborally. **Veronika**, you have a great energy that translates very well to being a researcher. A little petulance allows for critical analysis of data and a desire for success. Keep going; don't stop! **Caroline**, it has been nice having another night owl here at the lab, good luck getting your scholarship. **Christina**, thanks for being my yoga buddy, don't drown in work at your new lab.

Of course there are all the old members of the lab with whom I have had many a fika as well as good discussions. **Dhifaf**, **Erik**, **Giusy**, **Helena**, **Isabel**, **Riki**, **Renee**, **Bhavesh**, **Simona**, **Chiara**, **Takashi**, **CCJ** and **Regina**. Thank you.

<u>To the students:</u>

During my PhD time I resolved to have as many students as Rolf would let me take on. It turned out to be fewer than I thought it would be but they have been spectacular.

Madhura, as my first student I am glad that it was you whom I got to teach. You picked everything up immediately and within a couple months were teaching me. Your drive at work reignited my own motivation for our work. Together we shared many late nights in the lab, making the projects work. Thanks for all the help. **Kristina**, read above ☺. **Özcan**, I am glad you followed my advice and moved back to your girlfriend, I am sure that this has made life better for you both. You have a fantastic work ethic; together we made short work of two projects, incredible. Culturing 16 tumor cell lines and harmonizing the timing of them was very impressive, couldn't have done that myself.

In the Kiessling lab there have been many students passing through, and together we have had a great time here. **Tom**, **Maxi-Lu**, **Jochen**, **Sara**, **Mamud**, **Franziska**, **Aline**, **Ladan** and **Martin**. Thank you.

To my collaborators:

None of the work that I have done would have been possible without the help of an extensive network and group of collaborators.

Anna-Karin and Kenth, thank you for taking care of the mice for me. I have learned so much about how to handle and treat them from you.

Federica, **Laura and Stefania**, it has been a real pleasure working together with you. I loved that you were so enthusiastic and generated data so quickly for our collaboration. I hope we can work together in the future again.

Hinrich and Markus, you both have been fantastically kind and patient with me. It was a joy to come learn from you both in Germany a couple years ago. I hope that soon we can get our paper accepted and move on to the next promising project.

Nico, your stay here in Sweden was too short. I know that you hated the cold, and would be immensely disappointed with what we call summer, but I really enjoyed working and talking in Spanglish about the importance of platanoes together with you. Best of luck with your imminent dissertation.

Charlotte and Jeannette, I really enjoyed your energy at our lab meetings and am glad that we had the opportunity to work together.

Raja, when I first came to Sweden I remember that you gave this lecture about immunological methods, something that must not have been very interesting for you, but you presented with such passion and enthusiasm that I was blown away. I have attempted to adopt this style. Also, many thanks for taking the time to slog through my English.

Alexey and Alexey at MirImmune, it has been great working together with you guys. I really hope to be able to continue and expand our collaboration; there are so many ways to improve cancer immunotherapy yet.

Bruno, Gilles and Benoit at InCellArt, while our collaboration was not as fruitful as I imagined it would be it was great to work together with all of you. I'm sure that that nanotaxi will deliver many vaccines to patients in the future.

Bruce at Peregrine, I am very much looking forward to our collaboration and believe that we have a great opportunity here.

To the CCK administration:

Elle, Elizabeth, Jaun, Sören and Eva-Lena, thank you for keeping our labs running and for bearing with my requests for access to the fifth floor. Most of this thesis was inspired by the great view up there.

To the floor members:

The first floor of CCK has always had a great vibe. This is due in part by the great groups that are here. The Rolny group with **Majken** and **Tatjana**. The Mellstedt/Österborg group with **Amir**, **Mohammed**, **Salam**, **Barbro**, **Ingrid**, **Ann**, **Kia** and **Fariba**. The famous Dalianis group with **Tina**, **Torbjörn**, **Cecilia**, **Nathalie**, **Linea**, **Andreas**, **Lars**, **Anders** and **Nikos** (looking forward to your joining the Kiessling lab). The now missing Larsson group with **Vincent** (when are we going sailing again?), **Laia**, **Carl** and **Steve**. And the new Rassidakis group with **Nikos** and **Dimitrios**.

To my friends and family:

I would like to thank a couple friends in particular. The first I would like to acknowledge are **Huub** and **Gertie**, you were instrumental in making my transition to Europe and the Dutch university system as smooth as you could. Thank you for guiding me to Sweden. **Bastiaan**, you are a dude like none other, our times together have been great, thanks for the fun times. **Bleuenn and Mattieu**, its been great getting to know you guys, I can hardly believe it, but through Mindy I have made two friends outside of work. I look forward to teaching you guys a bit of sailing and playing boules on the outer archipelago islands.

Daniel en Ineke, thank you for welcoming me into your family, well, by now I think we really are family. It's always a pleasure to visit you in Wageningen, I love the curries and the way you both approach life. **Tom en Suzanne**, you guys are a ball of fun together and it's always a blast. **Maarten en Elodie**, I am glad that Mindy and I were able to visit in Australia this year, it was a great to see you in your new adopted natural habitats.

Pap en Mams, thank you for all your support. I could not have wished for a better pair of parents. The last couple of years I have finally come to an understanding about your crazy work ethic Pap, its great to care so much about something that you can dive in head first and not need to come up for air for weeks because you can breath underwater. I now know that I have inherited this trait from you. Unfortunately, I have also inherited the “wait until the last minute” trait. But I am working on that. Mam, you are the rock that keeps our family together, thank you for being there for all of us. I aspire to be able to pull our family closer together regardless of the thousands of miles. **Roland and Cameryn**, I want to thank you Roland for blazing a trail for me when we were younger, I could always look to you for where I should or shouldn’t go. I am always happy to see you two together, and I really enjoy talking to you, Cameryn, about all the things, thanks for that. **Arjan and Katie**, you are both incredible people, your drive to succeed is inspiring and your ability to make everything work well is crazy. **Theodore**, good luck buddy! If you ever need to a place to relax, give me a call. **Oma**, het is eindelijk zo ver oma, het studeren is voorbij! Ik vind het altijd fijn om bij jou langs te komen, en ben blij dat ik de laste jaren dit heb kunnen doen. Je hebt laatst een stroke gehad, en ik bewonder hoe je zo snel weer terug bent gekomen. Zo doen we dat hé Oma?

Mindy, I can’t believe it’s almost over. I get to come home to you! We have been flying back and forth for five years now, such a long time. Ik kijk er altijd naar uit dat we elkaar mogen zien, elke keer been ik weer helemaal blij als we samen zijn. It’s almost like we have been going on bi-weekly dates for years! Ook als we dan weer afscheid moeten nemen, komt er weer een traan in mijn ogen. Our daily skype/facetime calls kept our lives together and in sync. Zonder jou zou deze boekje hier niet liggen. Ik hou van jou liefje and I can’t wait to have more adventures together with you.

This work has been generously funded by the Radiumhemmets Forskningsfonder, the Swedish Cancer Society, the Swedish Medical Research Council, the Cancer Society of Stockholm, the ALF-project grant from Stockholm City Council, the Knut and Alice Wallenberg’s Foundation and the Karolinska-sponsored Center for Immune Modulatory Therapies for Autoimmunity and Cancer.

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Full title:
Co-expressed catalase protects chimeric antigen receptor-redirected T cells as well as bystander cells from oxidative stress-induced loss of anti-tumor activity.

Running title:
Catalase maintains CAR T cell anti-tumor activity

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Abstract

Treatment of cancer patients by adoptive T cell therapy has yielded promising results. In solid tumors, however, T cells encounter a hostile environment, in particular with increased inflammatory activity as a hallmark of the tumor milieu that goes along with abundant reactive oxygen species (ROS) that substantially impair anti-tumor activity. We present a strategy to render anti-tumor T cells more resilient towards ROS by co-expressing catalase along with a tumor specific chimeric antigen receptor (CAR) in order to increase their anti-oxidative capacity by metabolizing hydrogen peroxide (H_2O_2). In fact, T cells engineered with a bi-cistronic vector that concurrently expresses catalase (CAR-CAT) along with the CAR performed superior over CAR T cells as they showed increased levels of intracellular catalase and had a reduced oxidative state with less ROS accumulation in both the basal state and upon activation while maintaining their anti-tumor activity despite high H_2O_2 levels. Moreover, CAR-CAT T cells exerted a substantial bystander protection of non-transfected immune effector cells as measured by CD3ζ chain expression in bystander T cells even in presence of high H_2O_2 concentrations. Bystander NK cells, otherwise ROS-sensitive, efficiently eliminate their K562 target cells under H_2O_2-induced oxidative stress when admixed with CAR-CAT T cells. This approach represents a novel means for protecting tumor-infiltrating cells from tumor-associated oxidative stress-mediated repression.

Introduction

Tumor infiltrating lymphocytes (TILs) have long been recognized as a prognostic factor for cancer patients in a variety of tumor types (1). This has spurred the development of adoptive cell therapy with TILs, which in combination with non-myeloblative lymphodepletion regimens has resulted in some remarkable clinical response rates in metastatic melanoma patients (2, 3). Isolation and expansion of TILs from cancer patients is however not feasible for all tumor types, and genetic transfer of tumor specificity with T cell receptors (TCR) and chimeric antigen receptors (CAR) into T cells from peripheral blood is an attractive alternative. Similar to conventional T cells, the limitation of TCR transduced T cells are in their inability to recognize tumors that have down- regulated their MHC-class I molecules (4, 5). CARs circumvent this by providing specificity by a single chain fragment of a variable antibody region specific for a surface tumor antigen. CARs activate T cells through intracellular signaling domains such as CD3ζ which is improved by costimulation including CD28 or 4-1BB (6). Recently, transfer of such "second generation" CAR T cells targeting CD19 positive B cell lymphoid leukemia has shown encouraging clinical results in treating patients with bulky tumors (7-10). Though these results are galvanizing the field of adoptive cell therapy, clinical trials focusing on solid tumors have seen less success (11-13). The challenge for T cell based therapies of solid tumors lays in that T cells, in addition to reaching their targets, are required to survive and function within the unfavorable tumor microenvironment (TME).

Tumors cells have long been known to have high levels of oxidative stress and reactive oxygen species (ROS) which have been shown to play key roles in many aspects of tumorigenesis (14). Reactive oxygen intermediaries (ROI) and ROS, such as super oxide and hydrogen peroxide, are produced by all mammalian cells mainly as part of normal mitochondrial metabolic processes. Innate phagocytic immune cells produce high levels of ROS through the NADPH oxidase complex as their primary mechanisms of clearing bacterial infections. Oxidative stress exists when the balance between ROS production and antioxidant function is shifted in favor of ROS. Increased production of ROI in tumor cells can be attributed to alterations in metabolic pathways, as exemplified by glucose deprivation in breast carcinomas leading to decrease in intracellular pyruvate preventing decomposition of ROI (15).

Also tumor-infiltrating immune cells may be responsible for a large part of the ROS production. Thus immature myeloid cells found in tumors effectuate their suppressive function on the immune system via ROS (16, 17). Cancer patients have been found to have increased levels of activated granulocytes (18), subsequently defined as granulocytic myeloid derived suppressor cells (grMDSCs) (19). High concentrations of ROS can lead to necrotic cell death, although there is a window of ROS induced oxidative stress in which lymphocytes are still viable but become unresponsive (18). This has been linked to blockage of NF-κB activation due to protein oxidation, resulting in

deficient IFNγ, TNFα, and IL-2 production (20, 21). ROS induced alterations in T cell and NK cell functions may also be attributed to the decreased TCR-ζ and CD16-ζ chain levels found in tumor bearing patients and mice (22-24), which is associated with tumor accumulation of myeloid cells (25).

We have shown that T cells transduced with catalase (CAT) survive and function in toxic concentrations of H_2O_2 (26). To adapt the approach to cell therapy we aimed at enhancing persistence and function of tumor- redirected T cells in the environment of high oxidative stress. Here, we demonstrate that T cells modified with a bi-cistronic expression vector co-expressing the CAR as well as catalase (CAR-CAT) produce increased amounts of intracellular catalase and have a reduced intracellular oxidative state. This improves protection of the CAR-CAT transduced T cells from intrinsic oxidative stress, which is a result of T cell stimulation, as well as from extrinsic - especially tumor-associated - ROS. Such CAR-CAT T cells are able to lyse tumor cells in antigen specific manner under H_2O_2 induced oxidative stress, under which CAR T cells failed to do so. Furthermore, CAR-CAT T cells elicited a protective bystander effect allowing neighboring NK cells to kill tumor cells within a detrimental environment. CAR-CAT T cells provide a strategy to maintain anti-tumor activity of resident and adoptively transferred immune cells within the oxidative stress environment of tumors.

Materials and Methods

Cells and Reagents

HEK293T and SkoV3 cells were maintained in complete RPMI 1640 L-Glutamine+ medium supplemented with 10% (v/v) FCS, PenStrep (100 U/ml penicillin and 100 U/ml streptomycin), 1% MEM non-essential amino acids, 1mM Sodium Pyruvate and 50 µM 2-Mercaptoethanol (Life Technologies). PBMCs were obtained from healthy donors and used for transduction as well as the lymphocytic fraction from a healthy leukapheresis donor. These were either cultured in activation medium consisting of complete RPMI 1640 GlutaMAX with the addition of 500 U/ml IL-2 (Proleukin) or stimulation medium consisting of complete RPMI 1640 supplemented with 500 U/ml IL-2 and 100 ng/ml anti-CD3 (Orthoclone OKT3).

CAR engineering of T cells

T cells were transduced with recombinant retroviruses as described previously (27). T cells were purified from healthy donor PBMC followed by Pan T cell isolation (Miltenyi biotec) or by leukapheresis followed by elutriation and collection of the second fraction containing more than 95% lymphocytes. Elutriated lymphocytes were frozen down in 10% DMSO FCS and thawed prior to use. Lymphocytes were cultured in stimulation medium for two days then transferred to activation medium for four days. In parallel HEK293T cells were transfected with retroviral packaging plasmids pCOLT and pHIT60 along with a CAR expression vector using JetPrime (Polyplus). Activated lymphocytes were co-cultured with virus producing transiently transfected HEK293T cells for 48 hours. Transduction efficiency was analyzed by flow cytometry with goat F(ab)2 anti-human IgG PE (Southern Biotech) and mouse anti-human CD3-APC (BioLegend).

Catalase Detection

Transduced T cells were sorted using anti-PE MACs (Miltenyi biotec) beads in combination with F(ab)2 anti-human IgG PE (Southern Biotech). Sorted CAR positive lymphocytes were lysed in Cell Lytic M (Sigma-Aldrich) at a concentration of $1x10^6$ cells per 100µL. Protein concentrations of lysates were measured using Pierce BCA assay (Thermo Scientific). Catalase activity assays were performed as per manufactures instructions after normalization based on BCA assay (Life Technologies). To evaluate catalase protein expression western blots were performed as follows. 20 µg of lysate were loaded into 10% NuPAGE Bis-Tris acrylamide gels (Invitrogen) and run for 45 minutes at 200 V followed by transfer onto PVDF membrane for 3 hours at 40 V. Catalase was stained using 1:1000 dilution of rabbit α-human catalase (GenScript, A01202), actin was stained using mouse α-beta-actin at a dilution of 1:25,000 (Sigma-Aldrich) overnight at 4^{oC}. HRP linked α-rabbit and α-mouse antibodies were used for secondary antibodies (Cell Signaling Technology) and membranes were developed with ECL Prime Western Blotting Detection Reagent (GE Healthcare). Intracellular staining and detection of catalase was done using Cytofix/Cytoperm Kit (BD); catalase was stained using 2.5 µg/µl of rabbit anti-human Catalase antibody (GenScript) followed by anti-rabbit IgG FITC (Pharmingen) conjugated antibody.

Cell Death Assay
Lymphocytes were cultured in RPMI 1640 + L-glutamine supplemented with 500 U/ml IL2 at a concentration of $1x10^6$ cells per ml in the presence of H_2O_2. After 24 hours cells were washed with PBS and then stained with anti-human CD3-APC (BioLegend), 7-AAD (BioLegend) and AnnexinV-FITC (BioLegend) and acquired on BD LSRII.

Detection of thiols, reactive oxygen species and oxidative state
Cell surface thiols were evaluated using Alexa Fluor 488-C_5-maleimide (Life Technologies). Staining was done by washing cells thrice with 4^{oC} PBS followed by labeling with 5µM maleimide for 20 minutes at 4^{oC} in the dark. Cells were labeled with antibodies prior to acquisition on BD LSRII. Free reactive oxygen species were measured using L-012 luminol probe (Wako Chemicals GmbH). 0.5 mg/ml was the final concentration of L-012 in medium with cells. Luminescence was measured using (Centro LB 960) plate reader. CellROX (Invitrogen) was used to determine intracellular oxidative states. $1x10^5$ lymphocytes were loaded with 5 µM CellROX for short-term assays or 0.5 µM CellROX for long-term assays at 37^oC for 10 minutes. For basal ROS measurements, cells were incubated at 37^oC for the indicated duration followed by staining with anti-human CD3 PO (BioLegend) and F(ab)2 anti-human IgG PE (Southern Biotech) prior to acquisition with BD LSRII. To stimulate intracellular ROS, cells were activated by phorbol 12-myristate 13-acetate (PMA) (3 ng/µl) or dihydroxy-napthoquinone (DHNQ) (20 µM) for 2 hours prior to antibody labeling and acquisition by flow cytometry.

Cytotoxicity and Proliferation assay
Her2 positive SkoV3 cells were used as targets for Her2 specific CAR transduced T cells in standard chromium release assays. K562 were used as targets for NK cells. Target cells were loaded with ^{51}Cr (50µCi) (PerkinElmer) for one hour there after being washed of excess ^{51}Cr. Target cells were incubated for one hour in complete medium prior to being plated out into 96-well plates together with effector cells at different E:T ratios. Following co-culture with effector cells for 18 hours 25 µl of supernatant was transferred onto LumaPlates (PerkinElmer) and after desiccation were analyzed on the Micro-Beta scintillation (TRILUX 1450, PerkinElmer) plate reader. For proliferation, 5×10^3 T cells were seeded in a 96-well U bottom plate and stimulated to proliferate with 1µl anti-CD3/CD28 mAB0-coated beads per well. Every 24 hours cells were collected by Micro-Beta scintillation counter (TRILUX 1450, PerkinElmer) after pulsing with ^3H-thymidine (31µCi per well) (PerkinElmer) for 4 hours.

Results

CAR redirected T cells engineered with catalase.

Human peripheral blood T cells from healthy donors were engineered with a CAR specific for CEA or Her2 with or without a bi-cistronic cassette to co-express catalase. Catalase expressing CAR constructs (CAR-CAT) were based upon the CEA specific CAR BW431/26scFv-IgG1-CD28-CD3ζ and the Her2 specific CAR C6-B1.D2-IgG1-CD28-CD3ζ CAR (27, 28) by inserting the full-length human catalase cDNA downstream of the internal ribosome entry site (Figure 1A). Non-transduced T cells and T cells modified with a truncated NGFR specific CAR (Ctrl-CAR) lacking internal T cell signaling domains were used as controls. Retroviral transduction of these constructs into T cells gave efficiencies of approximately 55% CAR positive cells (Figure 1B). To determine the level of catalase present in the transduced T cells, CAR positive and negative cells were sorted from freshly transduced T cells using MACs beads specific for the CAR IgG linker region and the catalase recorded by Western blot analysis (Figure 1C). No differences were seen in the negative fractions of transduced T cells (Supplementary Figure 1A). To confirm the function of catalase in CAR positive cells, a catalase activity assay was performed on lysates from CAR, CAR-CAT, and Ctrl-CAR sorted cells. CAR-CAT transduced T cells were found to contain more than seven-fold higher catalase activity than CAR T cells without transduced catalase or Ctrl-CAR T cells (Fig 1D). Catalase activity was also measured in the lysate of CAR negative T cells and found to not to significantly differ between samples (Supplementary Figure 1B). Freshly transduced T cells were evaluated for their ability to neutralize H_2O_2. L-012, a ROS sensitive luminol (29)was added to T cells prior to addition of 50 μM H_2O_2. CAR-CAT T cells significantly reduced luminescence (Figure 1E) indicating reduced ROS activity in the presence of CAT Catalase was recorded in CAR-CAT T cells upon permeabilization indicating intracellular localization (Figure 1F).

CAR-CAT T cells display an increased antioxidant capacity against intrinsic ROS upon T cell activation

Activation of lymphocytes induces increased mitochondrial activity resulting in oxidative stress (30). We next asked if CAR-CAT T cells are more resistant to this type of cellular stress especially. Freshly transduced T cells were labeled with the oxidative stress indicator CellROX directly after transduction. The basal oxidative state was found to be lower in the CAR-CAT T cells compared to CAR T cells without transduced catalase (Figure 2A). This was also found to be the case after long-term culture (Figure 2B). We additionally stained cells with maleimide-alexa-488 and found that the difference between CAR-CAT and CAR T cells were minimal at 0 mM H_2O_2, but at 1 mM H_2O_2 CAR T cells were not able to maintain cell surface thiols with a MFI decrease of 20% while CAR-CAT maintained cell surface thiols with a decrease of 0.7%. A reduced oxidative state in the CAR-CAT T cells compared to CAR only transduced cells was also found after co-cultured for 24 hours with SkoV3 tumor cells (Supplementary Figure 2).

T cells encountering antigen at the tumor site will induce oxidative stress in the engaged T cells. To simulate this effect we stimulated CellROX labeled T cells by incubation with PMA or DHNQ (Figure 2C). CAR-CAT T cells displayed a decreased CellROX MFI compared to CAR T cells following PMA or DHNQ stimulation, similar to the differences found between their levels of basal oxidative states, indicating less oxidative stress of catalase engineered T cells upon activation.

CAR-CAT T cells maintain their activity under H_2O_2 stress

To examine if CAR-CAT transduced T cells were more resistant to high levels of oxidative stress cells were cultured in increasing concentrations of H_2O_2 (Figure 3A). T cells were freshly transduced and not sorted for CAR T cells. At 100 µM H_2O_2 CAR T cells were only 59% viable, which dropped to less than 30% viability at 200 H_2O_2. CAR-CAT T cells faired superior retaining their viability at 200 µM H_2O_2. Of note, activation of T cells for retroviral modification itself increased the resistance of T cells to oxidative stress compared to non-modified T cells.

Even if adoptively transferred T cells are able to survive in the presence of increased ROS levels, maintaining their function with respect to redirected cytolysis and amplifications remains crucial for their anti-tumor efficacy. When T cells recognize their target antigen they become strongly proliferative, as shown for CD19 specific CAR adoptively transferred into CLL patients (31). To address this issue we assayed the ability of T cells to proliferate at a level of ROS insult at which T cell viability is not affected. Transduced T cells were pulsed with 50 µM H_2O_2 and incubated with anti-CD3 and anti-CD28 beads for CAR independent stimulation. At this concentration of H_2O_2, CAR-CAT T cells still maintained their proliferative potential after 4 days compared to control T cells ($p < 0$ .005, Figure 3B).

We asked whether these cells retain their CAR endowed tumor specific effector functions. To address this, we studied the ability of Her2 specific CAR T cells to lyse the $Her2^+$ SkoV3 ovarian carcinoma cells under oxidative stress. CAR mediated specific lysis of SkoV3 cells was almost abolished in the CAR T cells at a concentration of 12.5 mM H_2O_2 , while the CAR-CAT transduced T cells efficiently lysed the SkoV3 cells (Figure 3C). Data demonstrate the superior capacity of CAR-CAT transduced T cells to maintain their tumor-specific cytotoxic function under conditions of oxidative stress where this function is lost in non-modified T cells.

CAR-CAT T cells mediate a protective bystander effect

The reduction of ROS by the CAR-CAT T cells may protect also non-modified immune cells in their vicinity. To investigate whether CAR-CAT T cells provide protection to bystanders, autologous non-modified T cells were stained by CellROX. CAR negative cells had decreased CellROX MFI for one hour as well as well as overnight staining (Figure 4A). To address whether this would be the case when oxidative stress was introduced, autologous T cells were labeled with CFSE and admixed with CAR or CAR-CAT engineered

lymphocytes. CAR-CAT bystander cells maintained basal oxidative stress level, while those admixed with CAR had increased oxidative stress (Figure 4B). Additionally, the level of surface thiols in CAR-CAT negative autologous T cells was reduced when compared to CAR negative autologous T cells (Figure 3C). The results showed that these non-transduced T cells co-cultured together with the CAR-CAT transduced cells had a reduced oxidative state when compared to their counterparts co-cultured together with CAR T cells. The supernatant had a five-fold increase in catalase activity in CAR-CAT T cell culture compared to from the supernatant of CAR T cells (Figure 4D). We conclude that CAR-CAT T cells released catalase in substantial amounts which reduced the oxidative state of co-cultured cells.

Intracellular oxidative stress, induced directly by the cancer cells or by immune-suppressive cells infiltrating the tumor lesion, can regulate T cell functions by reduingthe CD3-ζ making TCR mediated T cell activation less efficient (22, 32). We therefore studied if the CAR-CAT T cells, in a bystander fashion, could protect the non-transduced T cells from this detrimental repression. To simulate the situation, transduced and non-transduced T-cells were co-cultured, subjected to H_2O_2 insult and then stained for CD3-ζ. Exposure to H_2O_2 decreased of CD3-ζ in the non-modified T cells when co-cultured with non-modified or CAR modified T cells. In contrast, co-incubation with CAR-CAT T cells maintained CD3-ζ levels of bystander T cells (Figure 4E). In the third donor, no decrease in CD3-ζ was detected in bystander T cells (data not shown).

Tumor infiltrating lymphocytes also include NK cells which are sensitive to H_2O_2 induced inactivation (33). To address whether the CAR-CAT T cell mediated bystander effect is protective for NK cells in the near vicinity of the transduced T cells, NK cells were admixed with transduced T cells at a ratio of two transduced T cells to one NK cell and the cytolytic capability of the NK cells was assessed against K562 target cells under increasing concentrations of H_2O_2. In the absence of oxidative stress, there was no difference in NK cell mediated cytotoxicity against K562 co-cultured with CAR-CAT, CAR or Ctrl-CAR T cells (Fig 4F). In contrast, when exposed to increasing levels of H_2O_2, the NK cells co-incubated with CAR-CAT T cells were consistently more efficient in killing the K562 cells than NK cells co-cultured with CAR, Ctrl-CAR transduced or non-transduced T cells. We conclude that CAR-CAT T cells protect in trans both T and NK cells from oxidative stress mediated repression.

Discussion

Adoptive therapy with CAR modified T cells offers a powerful therapy for a variety of malignant entities. This has been realized for the treatment of hematological tumors such as CLL and ALL (9, 31); treatment of solid tumors, however, faces additional hurdles and needs further optimization (6). The stroma of solid tumors constitutes a barrier that actively suppresses the function of the adoptively transferred T cells by various immune suppressive mechanisms including mediators such as ROS, arginase, IDO, and PGE_2 (34, 35). In this study we demonstrate that redirected T cells, engineered to target tumor cells by a CAR specific for a cell surface antigen, can be protected from ROS induced oxidative stress by co-expressing catalase. The CARs used in this study are specific for Her2 and CEA, respectively, and have been shown to eliminate tumor cells with the respective targets in a specific fashion (28, 36). The Her2 or CEA are highly expressed in a variety of breast cancer and colorectal cancer (CRC) lesions, respectively (37, 38), both of which have increased ROS production and local oxidative stress (15, 39). In CRC, oxidative stress promotes proliferation of tumor cells while being insufficient to cause cell death (40). Carcinoma infiltrating lymphocytes have experienced high levels of oxidative stress as measured by 8-OHdG staining (39). Also breast cancers have high levels of oxidative stress, being a driving factor in breast cancer progression. Accordingly, lymphocytes from breast cancer patients exhibit increased oxidized DNA levels as compared to healthy donor lymphocytes (41). Interestingly, when reducing oxidative stress in aggressive breast cancer tumors are sensitized to chemotherapy (15). This ROS feature of both cancer types provides the rationale for a potentially beneficial effect of co-expressing catalase in CAR transduced T cells homing to these tumors.

We revealed that co-expressing catalase in CAR T cells allowed for a reduced oxidative state in engineered T cells, an effect that remained when cells were activated by TCR/CD3 engagement or while co-cultured with tumor cells. A reduced oxidative state is essential for maintaining T cell function in the long-term. This is particularly of clinical relevance in the setting of adoptive cell therapy where the transplanted T cells are thousand fold expanded *ex vivo* prior to transfer and entering the tumor tissues. T cell subsets are differentially affected by ROS, particularly CD8+ T effector memory cells being more susceptible to ROS induced cell death and loss in function than their naïve T cell counterparts (21). These memory cells are essential for providing a better clinical outcome in CRC patients (42).

The tumor tissue is infiltrated with a large number of immune cells, most of which are not of lymphoid but of myeloid origin. These macrophages, monocytes, granulocytes and myeloid derived suppressor cells (MDSCs) produce ROS and thus suppress the lymphoid anti-tumor immune response. Activated infiltrating granulocytes in particular inactivate T cells, and addition of ROS scavengers was able to rescue their function (18). MDSCs are potent producers of ROS, mainly through the activation of the NOX2 pathway leading to the production of super oxide, and have been shown to exert some of their suppressive function through this pathway (17). High catalase activity in those

tumor targeting lymphoid cells upon transgenic catalase expression provides a strategy to resist ROS mediated repression in the tumor tissue.

In patients, CAR transduced T cells are able to clear large tumor burdens, sometimes leading to tumor lysis syndrome (8). We confirmed the high efficacy of the CAR transduced T cells in cytotoxicity assays. CAR-CAT T cells retained their ability to lyse Her2 positive tumor cells under conditions of oxidative stress, while this ability was lost in control CAR T cells. Furthermore, 50 µM H_2O_2 affected the capacity of control T cells and CAR T cells to proliferate in response to a strong proliferative stimulus while CAR-CAT transduced T cells retained proliferative capacity (Figure 3B). We conclude that genetically modified T cells which over-express catalase resist oxidative stress to certain levels which may be sufficient to induced remain functional upon entering the tumor stroma.

Protecting tumor infiltrating T and NK cells from ROS mediated inactivation would maintain their anti-tumor activity, the latter cells attacking those cancer cells which lack the particular tumor-associated antigen recognized by redirected T cells. In line with this concept we found that CAR-CAT T cells were capable of reducing the oxidative state of bystander T cells. Under these conditions NK cells are enabled to execute their anti-tumor response. This bystander effect was likely mediated by the catalase present in the supernatant of CAR-CAT T cells. We showed that T cells engineered with CAR and catalase preserved CD3-ζ levels of the bystander T cells (Fig 4E). This may result in more efficient tumor elimination, including of cancer cells lacking CAR targeted antigens. Protecting bystander immune cells in trans by catalase engineered T cells may thereby indirectly provide a benefit in the therapy of solid tumors. In addition to CAR redirected T cells themselves, tumor infiltrating lymphocytes, which are inactivated by ROS producing stroma cells may become reactivated when ROS induced immunosuppression is removed. Increased oxidative stress decreases TCR/CD3ζ expression in T cells inhibiting their TCR mediated effector functions. In cancer patients CD3ζ is often down regulated in tumor infiltrating T cells, accompanied by loss of cytolytic activity as well as loss of proliferative potential (23, 32, 43). In gastric carcinoma, the five year survival of patients was significantly improved when TILs maintained normal levels of CD3-ζ expression (44), underlining the therapeutic potential in sustaining function of infiltrating immune cells in a ROS mediated immune repressive environment.
Due to their high sensitivity towards oxidative stress, intra tumor activity of NK cells is likely compromised (45), particularly of the cytotoxic CD56 dim NK cell subset (33). We found Data indicate that the bystander effect of CAR-CAT T cells extended also to NK cells and rescued their cytolytic ability at high H_2O_2 concentrations (Fig 4F). Beyond this, CAR-CAT T cells are able to modify the suppressive cells in the tumor tissue. MDSCs require high levels of ROS to retain their suppressive phenotype; in absence of ROS immature MDSCs differentiate into non-suppressive monocytes (17) which, together with other mechanisms, finally may result in a global change in the immune surveillance of cancer.

The approach of combining tumor redirected CAR T cells with the transgenic expression of molecules that modulate the oxidative state in the tumor milieu may be extended to several other categories of molecules which counteract T cell function such as arginase-1, IDO or iNOS. Additionally, other strategies to reduce tumor immunosuppression, for example by co-engineering T cells with TGF-β dominant negative receptor have shown impressive results(46) and suggest that targeting these suppressive mechanisms may be essential to improving T cell based therapies(6). Our data imply that the strategy to target ROS may improve both the antigen- specific and antigen-independent tumor elimination, resulting in a more rapid and efficacious tumor elimination which likely improves the outcome of adoptive T cell therapy of cancer.

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Footnotes:

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Footnote:
Research described here have been supported by grants to R.K. from The Swedish Medical Research Council (K2011-66X-15387-07-3 VR), The Swedish Cancer Society (12 0598 Cancerfonden), The Cancer Society of Stockholm (121103 Cancerföreningen, Radiumhemmets Forskningsfonder), ALF-Project grant from Stockholm City Council (20110070 ALF Medicin 2012).
Work in the H.A. laboratory was supported by the Else Kröner-Fresenius Stiftung, the Sander-Stiftung and the Deutsche Krebshilfe.

Figure Legends:

Figure 1. Design of bicistronic expression vector for chimeric antigen receptors and catalase expression. (A) Schematic diagram depicting the two sets of CAR used. (B) PBMC from healthy donors were cultured for a four days and transduced using either bicistronic retroviral expression vectors for CAR and catalase or retroviral expression vector for CAR alone. Expression of CAR on transduced T cells was assessed by staining with PE conjugated F(ab)2 anti-human IgG that binds to the extracellular FC region of the CAR and APC-conjugated anti-CD3. PE conjugated isotype antibody were used to confirm lack of non-specific binding. CAR cells were gated on lymphocyte population in FSC and SSC prior to gating CAR positive cells. (C) Protein lysates from MACs sorted transduced T cells were analyzed by western-blot. Relative protein expression was determined by ImageJ analysis of the intensity of the bands from the western blot. (D) MACs sorted lysates were used to measure catalase activity. (E) Luminescence from 10^5 transduced or non-transduced cells was measured after adding L-012 and H_2O_2. (F) Transduced T cells were perimabilized and rabbit polyclonal anti-human catalase antibody was used to stain for intracellular catalase. FITC conjugated anti-rabbit IgG antibody was used to analyze the samples with flow cytometry. Data are presented as means ± SD, *** $P < 0.005$ by students T-test using GraphPad 5.

Figure 2. Oxidative state is reduced in CAR-CAT T cells compared to CAR T cells. (A) Directly after co-culture with HEK293T cells, transduced T cells were labeled with CellROX (5µM) in complete medium. (B) After short-term one-hour culture, or long-term 18 hr culture, cells were labeled with PE anti-human IgG and Pacific Orange anti-human CD3 and samples were acquired on BD-LSR2. (C) To induced oxidative stress, freshly transduced T cells were loaded with CellROX and stimulated with PMA (3µg/ml) and DHNQ (20µM) for two hours before acquiring samples by flow cytometry. Samples were analyzed using FlowJo.

Figure 3. CAR-CAT T cells maintain viability and functionality under H_2O_2 induced oxidative stress. (A) After transduction with CAR or CAR-catalase T cells were re-suspended to 2 x 10^5 in 200 µl complete medium RPMI containing 10% FCS and exposed to increasing concentrations of H_2O_2. After 24 hrs cells were stained with AnnexinV-FITC and 7AAD and analyzed by FACs. (B) 50µM H_2O_2 was used to induce oxidative stress in 5 x 10^3 engineered or non-modified T cells being stimulated with CD3-CD28 proliferation beads for four days and cell proliferation was measured by ^{3}H-thymidine incorporation for 4 hrs. (C) Transduced T cells were used as effectors for targeting Her2 positive tumors at an E:T ratio of 1:2. Freshly transduced T cells were cultured overnight with increasing concentrations of H_2O_2 to induce oxidative stress. After 18 hrs target cells were labeled with ^{51}Cr and transferred into effector cell containing wells. Supernatant was collected after 24 hr co-culture and transferred to LumaPlates and read out on MicroBeta. The percent specific lysis was calculated using the following formula: $(CPM_{sample} - CPM_{spontaneous})/(CPM_{maximum} - CPM_{spontaneous})$. Data are

presented as means ± SD, ** P < 0.005 by 2 way ANOVA using GraphPad 5 for B and C between CAR and CAR-CAT.

Figure 4. A bystander effect is mediated by CAR-CAT T cells towards non-modified T and NK cells. (A) T cells were labeled with CellROX for both a short-term and long-term staining and CellROX MFI on CAR negative T cells was determined after cells were acquired by FACs. (B) Healthy donor autologous T cells were CFSE labeled and admixed with transduced T cells followed by staining with antibodies and CellROX and acquired by flow cytometry. (C) CAR-CAT and CAR negative cells were labeled with maleimide and surface thiols were evaluated. (D) Supernatants were collected from transduced T cells and non-transduced and tested for catalase activity. (E) H_2O_2 was used to induce oxidative stress in T cell culture for two hours prior to staining. 2 x 10^5 cells were stained for CD3-ζ using FITC conjugated anti-CD3-ζ after permiabilization with 0.25% PFA and Digitonin. Cells were acquired by FACs and change in CD3ζ MFI was calculated by: CD3ζ $MFI_{(X\mu M\ H2O2)}$ – CD3ζ $MFI_{(0\mu M\ H2O2)}$ gated on the CAR negative fraction. (F) NK cells were co-cultured with engineered T cells at a ratio of 2:1 CAR T cell to NK cell overnight under oxidative stress induced by different concentrations H_2O_2. K562 target cells were loaded with ^{51}Cr and added to the NK/T cell mix after H_2O_2 co-culture at a ratio of 1:1 NK cell to K562. 25µl of supernatant was transferred to LumaPlates and read out on MicroBeta. Data are presented as means ± SD, * P < 0.05 by 2 way ANOVA using GraphPad 5 for C between CAR and CAR-CAT.

Figure 1

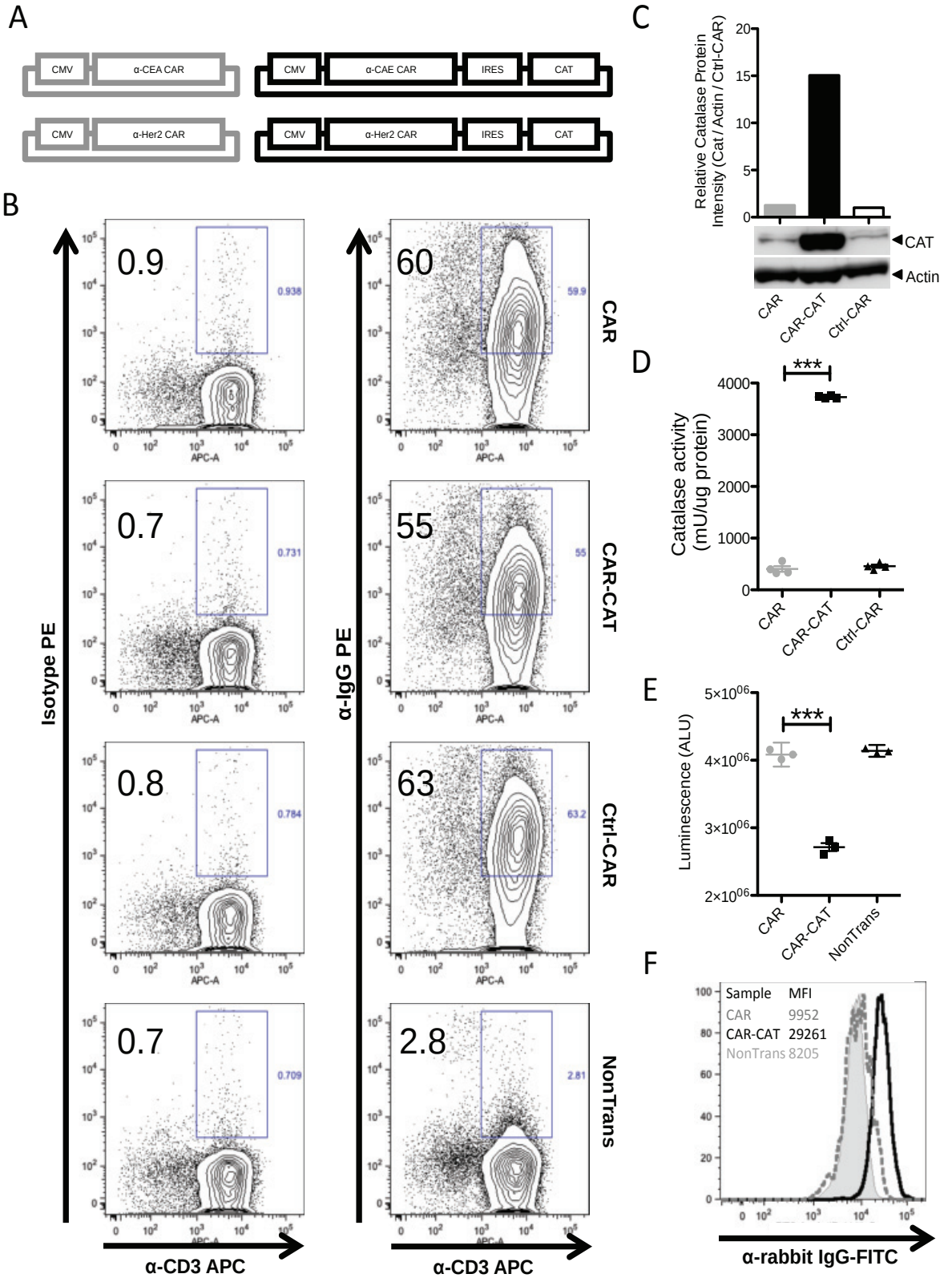
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Figure 2

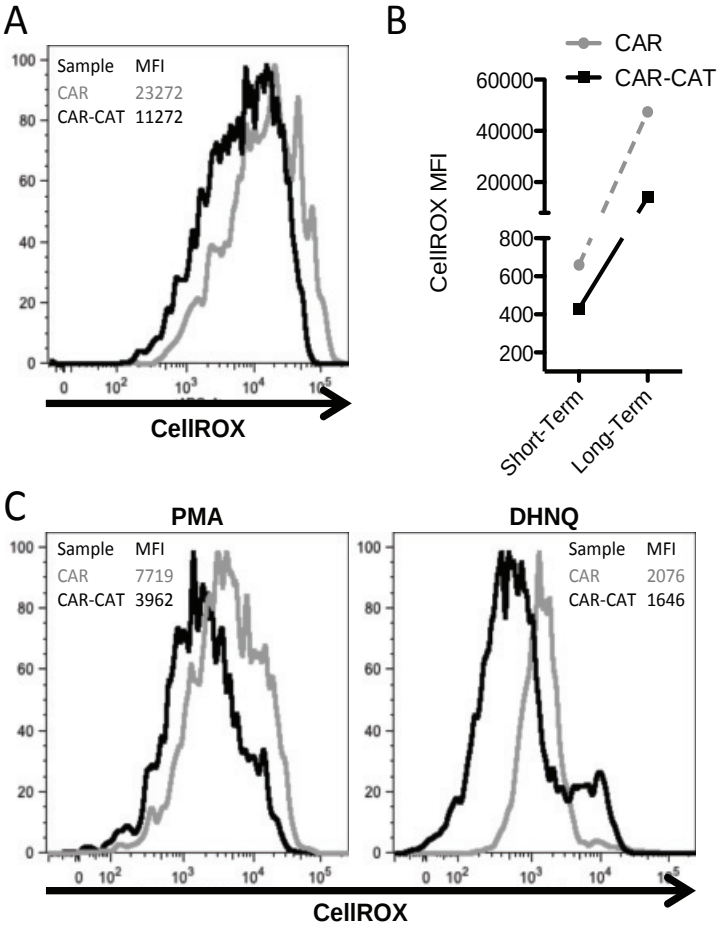
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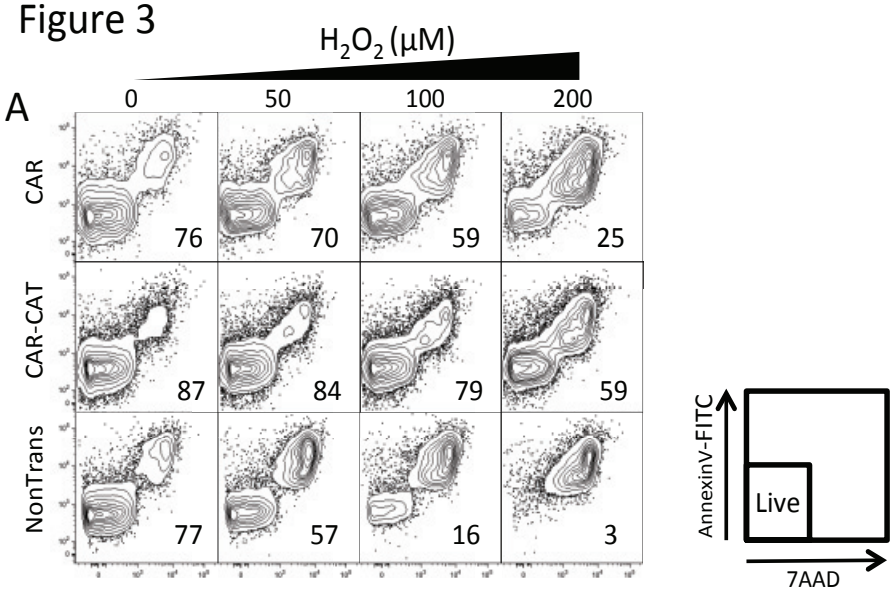
Figure 3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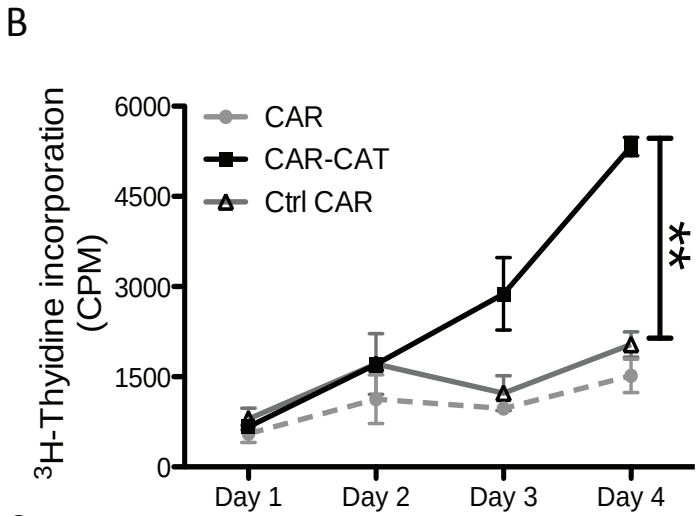
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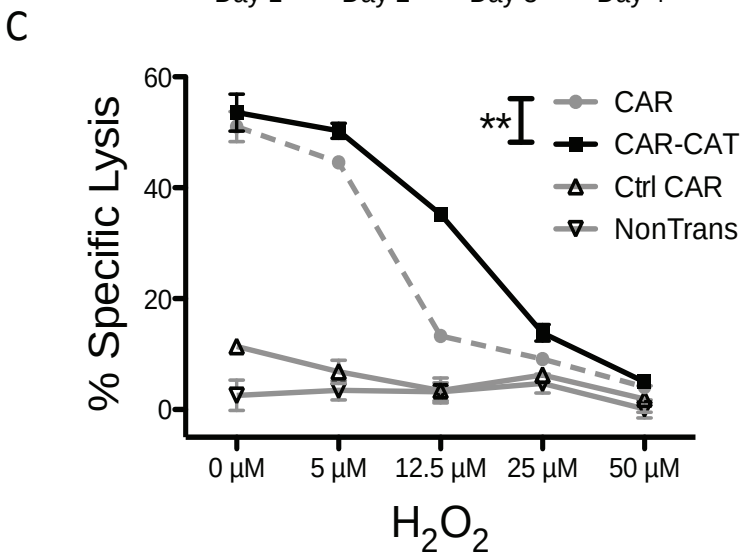
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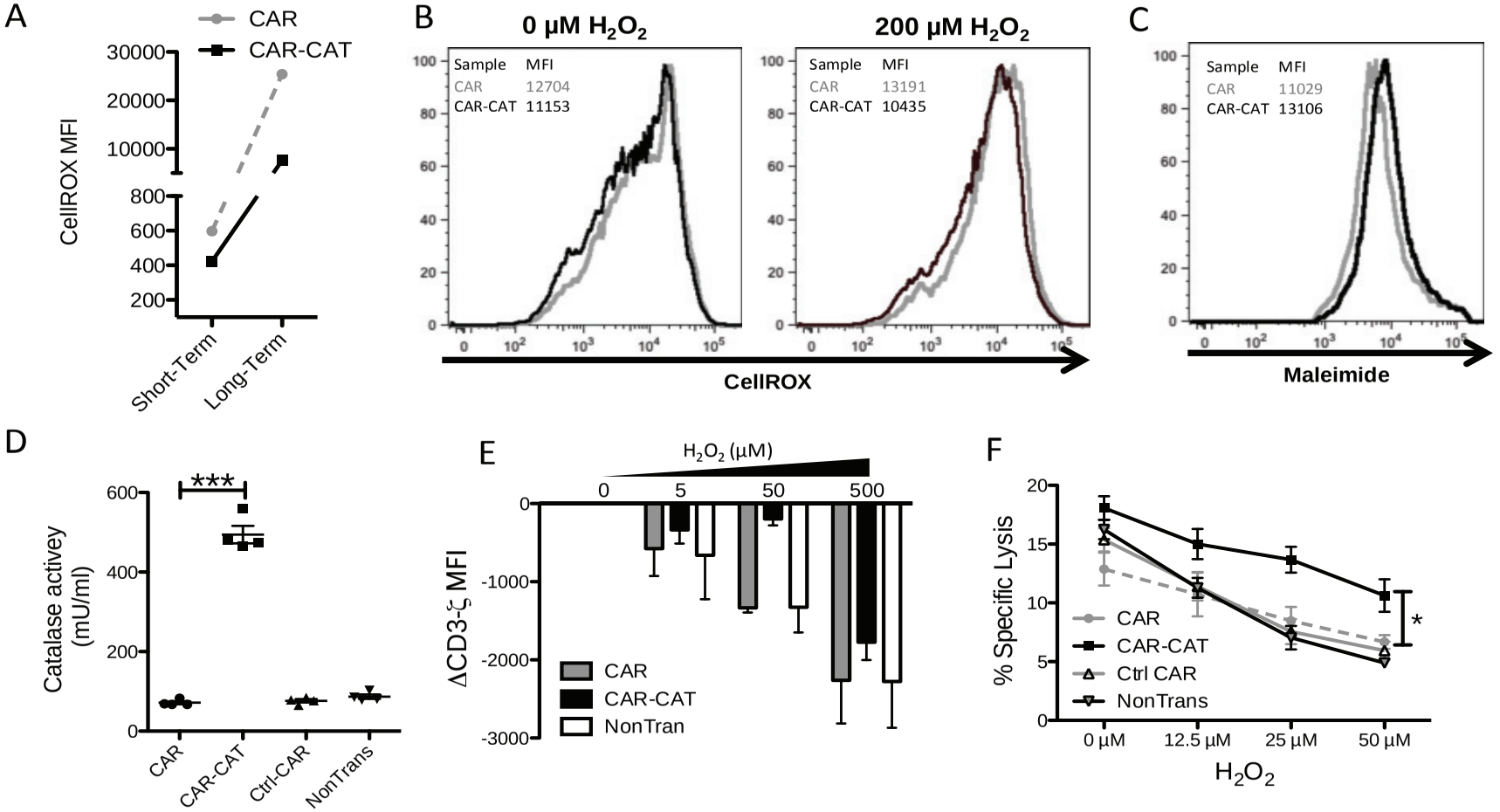
Figure 4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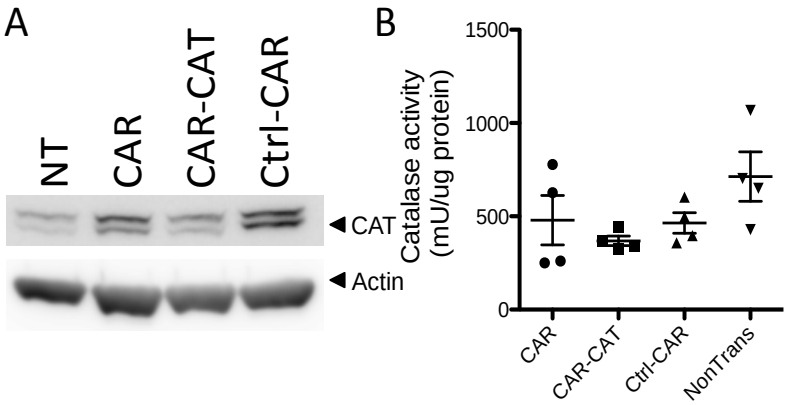
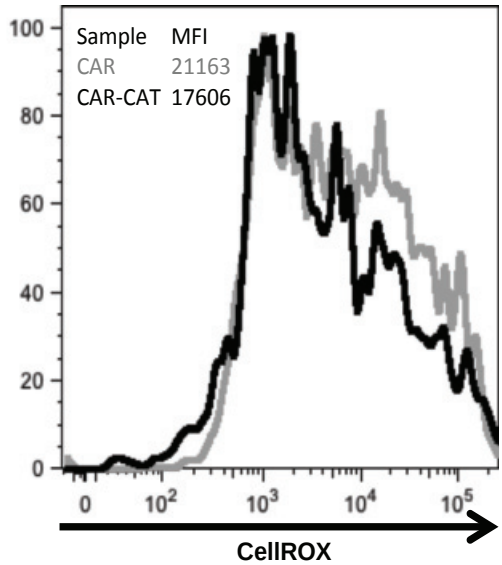


Supplementary Figure 1. CAR negative cells do not have increased catalase activity. (A) Lysates were prepared from the MACs sorted negative fraction after CAR positive cells had been selected out. Catalase activity was assessed in these lysates. (B) Similarly, the CAR negative fraction lysates were loaded into western blot and evaluated for catalase protein.



Supplementary Figure 2. Catalase co-expressing T cells maintain a lower oxidative state in the presence of tumor. $5x10^4$ Transduced T cells were co-cultured $1x10^5$ SkoV3 cells for 24 hours followed by labeling with CellROX and antibodies prior to acquisition by flow cytometry.

II

RESEARCH PAPER

Human Vaccines & Immunotherapeutics 9:10, 2189–2195; October 2013; © 2013 Landes Bioscience

NF-κB activation during intradermal DNA vaccination is essential for eliciting tumor protective antigen-specific CTL responses

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Keywords: DNA vaccine, intradermal, electroporation, type-I interferon, NF-κB, tumor immunity, cytotoxic T lymphocyte (CTL)

DNA vaccines have been shown to elicit tumor-protective cytotoxic T lymphocyte (CTL) immunity in preclinical models, but have shown limited efficacy in cancer patients. Plasmids used for DNA vaccines can stimulate several innate immune receptors, triggering the activation of master transcription factors, including interferon regulatory factor 3 (IRF3) and nuclear factor κ B (NF-κB). These transcription factors drive the production of type I interferons (IFNs) and pro-inflammatory cytokines, which promote the induction of CTL responses. Understanding the innate immune signaling pathways triggered by DNA vaccines that control the generation of CTL responses will increase our ability to design more effective vaccines. To gain insight into the contribution of these pathways, we vaccinated mice lacking different signaling components with plasmids encoding tyrosinase-related protein 2 (TRP2) or ovalbumin (OVA) using intradermal electroporation. Antigen-specific CTL responses were detected by intracellular IFN-γ staining and in vivo cytotoxicity. Mice lacking IRF3, IFN-α receptor, IL-1β/IL-18, TLR9 or MyD88 showed similar CTL responses to wild-type mice, arguing that none of these molecules were required for the immunogenicity of DNA vaccines. To elucidate the role of NF-κB activation we co-vaccinated mice with pIκBα-SR, a plasmid encoding a mutant IκBα that blocks NF-κB activity. Mice vaccinated with pIκBα-SR and the TRP2-encoding plasmid (pTRP2) drastically reduced the frequencies of TRP2-specific CTLs and were unable to suppress lung melanoma metastasis in vivo, as compared with mice vaccinated only with pTRP2. Taken together these results indicate that the activation of NF-κB is essential for the immunogenicity of intradermal DNA vaccines.

Introduction

Vaccinations have been an essential part of basic medical care for over a century, providing protection to various diseases and pathogens. Traditionally inactive or attenuated viruses have been used to establish humoral and cellular immunity. Since the advent of molecular biology and the ease of DNA manipulation, it has been proposed to use DNA as a vehicle of immunization.[1] Different techniques have been developed to establish immunity against DNA-encoded antigens (Ag). Intramuscular and intradermal (i.d.) injections of naked DNA followed by electroporation (EP) greatly increases the ability of DNA vaccines to elicit cellular immune responses including cytotoxic $CD8^+$ T lymphocytes (CTL).[2,3] Cancer vaccines able to elicit strong antigen-specific CTL responses that are effective at eliminating tumors are highly desired for achieving favorable clinical outcomes.[1,4,5]

Mature activated antigen presenting cells (APC), such as dendritic cells (DC) are essential for establishing effective CTL responses. DNA vaccines can target sentinel APCs, such as Langerhans cells and dermal DCs, that are proficient in processing the DNA-encoded antigen and migrating to lymphoid organs to elicit Ag-specific T and B cell responses.[2,3,6] Skin-resident APCs are well suited to recognize pathogen-associated molecular patterns (PAMPs) such as double stranded plasmid DNA used for DNA vaccines. Pattern recognition receptors (PRRs), such as Toll like receptors (TLRs), present mostly in APCs detect PAMPs and trigger signaling pathways that activate master transcription factors, including interferon regulatory factors (IRF) and nuclear factor κ B (NF-κB). The resulting production of type I interferons (IFNs), proinflammatory cytokines and chemokines promotes DC maturation that in turn generate CTL responses linking innate to adaptive immune responses. TLR9 recognizes DNA containing unmethylated CpG motifs from bacterial and viral DNA present within the endosomal compartment.[7] Cytosolic DNA sensors include DExD/H box helicases (DHX9/36),[8] DNA-dependent activator of IFN regulatory factors (DAI),[9] absent in melanoma 2 (AIM2),[10] meiotic recombination 11 homolog A (MRE11),[11] gamma-interferon-inducible

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Submitted: 03/25/2013; Revised: 06/25/2013; Accepted: 07/10/2013
http://dx.doi.org/10.4161/hv.25699

protein 16 (IFI16),[12] leucine rich repeat flightless-interacting protein (LRRFIP1),[13] probable ATP-dependent RNA helicase DDX41[14] and RNA polymerase III,[15] which transcribes dsDNA into double stranded RNA leading to RIG-I activation. However, which of these DNA-sensing signaling pathways promotes the maturation of APCs after DNA electroporation leading to the generation of CTL responses is not fully understood, especially for intradermal vaccination. Understanding the innate immune mechanisms involved in this process will increase our capacity to design more potent DNA vaccines.

In the following study, we explored the mechanisms involved in the induction of CTL responses elicited by intradermal DNA electroporation resulting in tumor protection. INF-α and transcription factor IRF3, previously identified as a key signaling pathway for establishing response to DNA vaccines, were not required for the induction of CTL responses by intradermal DNA vaccination. Furthermore, the generation of CTL responses was found to be independent of the classical TLR9 DNA sensing pathway and any other MyD88-dependent proinflammatory pathway. Lastly, we demonstrate that NF-κB acts as a master transcription factor that is essential for inducing antigen-specific CTLs and in providing protection against in vivo tumor challenge.

Results

Type 1 interferons are not required for intradermal DNA vaccine-induced CTL responses. DNA-sensing PRRs, including TLR9 and cytosolic DNA sensors, can trigger the production of type I IFNs, which are an important bridge between innate and adaptive immunity by promoting APC maturation and induction of T cell responses.[17,18] Moreover, type I IFNs have been found to be essential for the immunogenicity of DNA vaccines delivered intramuscularly in a TLR9-independent fashion.[19,20] To determine whether type I IFNs are also essential in establishing CTL immunity after intradermal DNA electroporation, wild type (WT) and IFNαR$^{-/-}$ mice on the 129sv background were immunized with the plasmid encoding the model antigen ovalbumin (OVA). In contrast to previous studies, CTL responses measured after the second immunization in peripheral blood from IFNαR$^{-/-}$ mice were not reduced compared with WT mice (**Fig. 1A**). To test whether the effector function of elicited CTLs was impaired in IFNαR deficient mice, an in vivo cytotoxicity assay was performed. Vaccinated mice were adoptively transferred with CFSE^{high} and CFSE^{low} labeled splenocytes pulsed with OVA and control peptides, respectively. Flow cytometry analysis of lymph nodes from recipient mice showed that killing of CFSE^{high} labeled OVA peptide pulsed lymphocytes was not significantly different between WT and IFNαR$^{-/-}$ mice ($p = 0.19$), confirming the establishment of functional OVA-specific CTL immunity (**Fig. 1B**).

Unlike OVA, tumor antigens are poorly immunogenic and induction of CTL responses against such antigens is controlled by diverse mechanisms of tumor-associated self-tolerance. To identify if the strong immunogenicity of OVA was the reason for the vital CTL responses observed regardless of the deficiency in type I IFN signaling, mice were vaccinated with a vaccine encoding the melanoma antigen tyrosinase-related protein 2 (TRP2). No significant differences in TRP2-specific CTL frequencies could be found between IFNαR$^{-/-}$ and WT mice (**Fig. 1C**). To further confirm these results, the role of IRF3, a key transcription factor driving type I IFN expression resulting after ligation of cytosolic DNA sensors, such as DAI and IFI16, was examined. IRF3$^{-/-}$ mice were able to generate CTL responses as strong as WT mice after vaccination with the TRP2-encoding plasmid (**Fig. 1D**). These results demonstrate that type I IFN/IRF3 signaling axis is not required for the ability of intradermal DNA vaccines to elicit antigen-specific CTL responses.

TLR9 and MyD88 do not mediate the generation of CTL responses after intradermal DNA vaccination. Plasmid DNA produced in the gram-negative bacteria *E. coli* contains unmethylated CpG motifs that could activate TLR9 and downstream signaling pathways through the adaptor protein myeloid differentiation primary response gene 88 (MyD88), which functions as a central adaptor protein for other TLRs (except TLR3) and the DNA sensor DHX9.[21] This led us to speculate that TLR9-mediated innate immune activation could possibly contribute to the specific adaptive CTL responses elicited by intradermal DNA vaccination. To determine if this was indeed the case, TLR9$^{-/-}$, MyD88$^{-/-}$ and WT mice on C57BL/6 background were vaccinated, as described above, with the OVA-encoding plasmid. Vaccination generated levels of OVA-specific IFN-γ producing CTLs that were similar between the three groups (**Fig. 2A**). To confirm the functionality of the CTLs generated, in vivo cytotoxicity was tested, demonstrating that cytotoxic potential of OVA-specific CTLs was intact in TLR9 and MyD88 KO mice as compared with wild-type mice. In both cases, there was no significant difference in the OVA-specific killing between KO mice and wild-type mice ($p = 0.75$ and $p = 0.98$ respectively) (**Fig. 2B**). Similar results were obtained when the less immunogenic TRP2 antigen was tested in these TLR9, MyD88 KO models. TRP2-specific CTL frequencies were not different between wild-type and KO mice (**Fig. 2C**), indicating that TLR9 and downstream MyD88-dependent signaling are not required for the generation of CTL responses elicited by i.d. EP DNA vaccines. A different proinflammatory pathway that can contribute to the immunogenicity of DNA vaccines is the inflammasome. AIM2-mediated DNA sensing can trigger inflammasome activation, leading to the activation of caspase-1 and the catalytic cleavage of pro-forms of the pro-inflammatory cytokines IL-1β and IL-18 into their active secreted form. Given the observed high expression of the inflammasome protein AIM2 in skin,[22] we studied whether IL-1β$^{-/-}$ IL-18$^{-/-}$ mice were able to mount similar CTL responses as WT mice. We found that intradermal electroporation induced similar specific CTL responses between the KO and WT mice measured as IFN-γ producingCTLs (**Fig. 2D**) and in vivo cytotoxicity (**Fig. 2E**).

Suppression of NF-κB during intradermal DNA vaccination decreases CTL responses and impedes tumor rejection. Most DNA-sensing PRR (DAI, IFI16, AIM2, DHX9) signaling pathways converge in the activation and nuclear translocation of NF-κB to drive the expression of proinflammatory transcriptional targets. To investigate the role of this pathway, we used a plasmid encoding the IκBα-super repressor (IκBα-SR), which functions similarly to endogenous IκBα in its ability to bind and

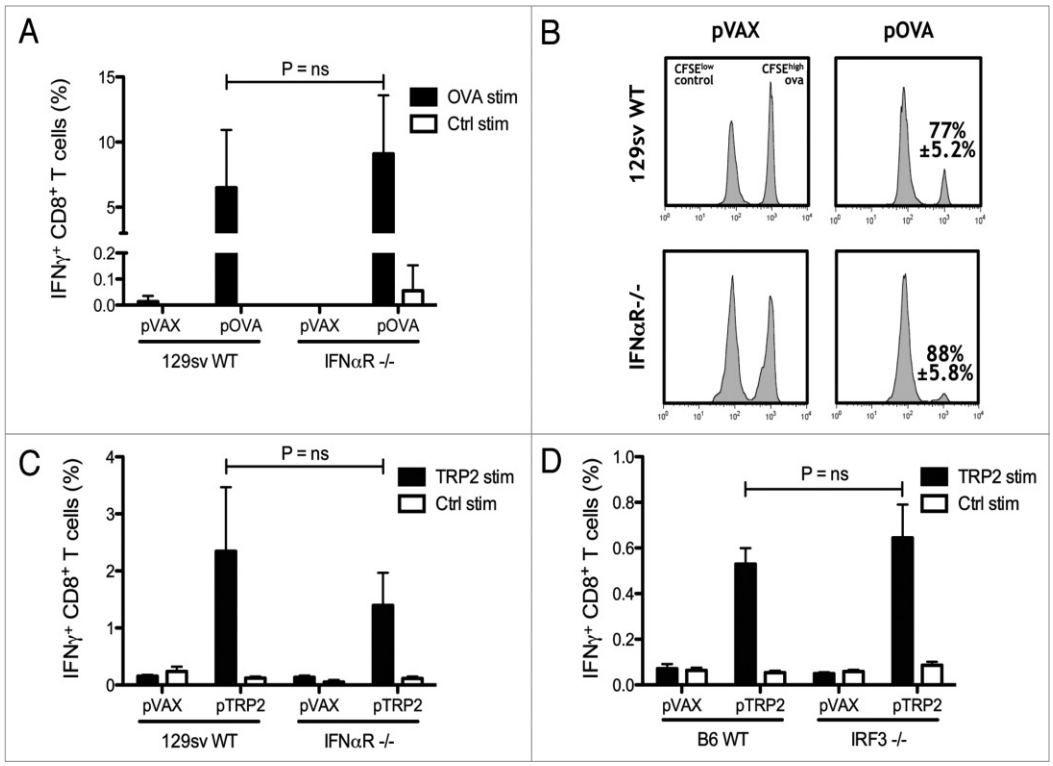

Figure 1. Type 1 interferons are not required for generating CTL responses. Wild-type (WT) and IFNαR$^{-/-}$ mice on 129sv background were vaccinated twice with the OVA-encoding plasmid pOVA. The percentages of IFN-γ producing CD8$^+$ T lymphocytes were detected after in vitro stimulation with OVA and control peptides (**A**). In vivo cytotoxicity was measured by quantifying OVA pulsed CFSE^{High} labeled target cells and compared with control pulsed CFSE^{Low} labeled cells in WT and IFNαR$^{-/-}$ mice vaccinated with pOVA. The mean percentage ± SEM of specific killing for each case is indicated (**B**). Tyrosinase-related protein 2 (TRP2) encoding DNA was used to vaccinate WT and IFNαR$^{-/-}$ 129sv mice. IFN-γ producing CD8 T lymphocytes were measured after in vitro stimulation with TRP2 and control peptides (**C**). WT and IRF3$^{-/-}$ mice on C57BL/6 background were vaccinated with TRP2-encoding plasmid and the percentages of IFN-γ-producing CD8 T cells were measured by flow cytometry after in vitro stimulation with TRP2 and control peptides (**D**). Bars indicate the mean ± SEM.

block NF-κB, but carries mutations that prevent its phosphorylation and subsequent degradation and, as consequence, stably impedes the translocation of NF-κB to the nucleus.[23] Thus, this plasmid allows blocking of NF-κB activation specifically at the vaccination site in the context of DNA vaccines.[24] To test the activation of NF-κB during DNA vaccination, a NF-κB luciferase reporter plasmid was i.d EP into the hindquarters of mice as done with DNA vaccines. In vivo luciferase activity was detected six hours after intradermal electroporation, which is indicative of early NF-κB activation. As expected, when the functionality of IκBα-SR was evaluated, co-electroporation with the IκBα-SR encoding plasmid showed decreased in vivo bioluminescence (**Fig. 3A**). To test the ability of NF-κB activation during intradermal DNA vaccination to mediate specific CTL antitumor responses, mice were vaccinated with the TRP2–encoding plasmid and pIκBα-SR. This led to a stunning decrease in IFN-γ producing CD8$^+$ T lymphocytes as compared with mice vaccinated with pTRP2 alone (**Fig. 3B**).

Finally to confirm that the reduced TRP2-specific CTL responses would have an impact on the ability of intradermal DNA vaccination to confer tumor protection, the B16F10 mouse melanoma lung metastasis model was used. Mice were vaccinated with either control plasmid, pTRP2 or co-vaccinated with pTRP2 and IκBα-SR encoding plasmid as described above; 14 d after the second immunization were challenged with 10^5 B16F10 cells injected i.v. Three weeks after the challenge, lungs were excised and the melanoma metastatic foci were enumerated (**Fig. 3C**). Accordingly, significantly decreased melanoma metastasis was observed in mice vaccinated with TRP2 encoding plasmid, whereas mice co-electroporated with both pTRP2 and pIκBα-SR were unable to suppress melanoma formation. Note that electroporation with the IκBα-SR encoding plasmid did not affect lung metastasis when administrated by itself (data not shown). In conclusion, these results indicate that the activation of NF-κB during intradermal DNA vaccination is essential for the induction of CTL-mediated tumor protective immunity.

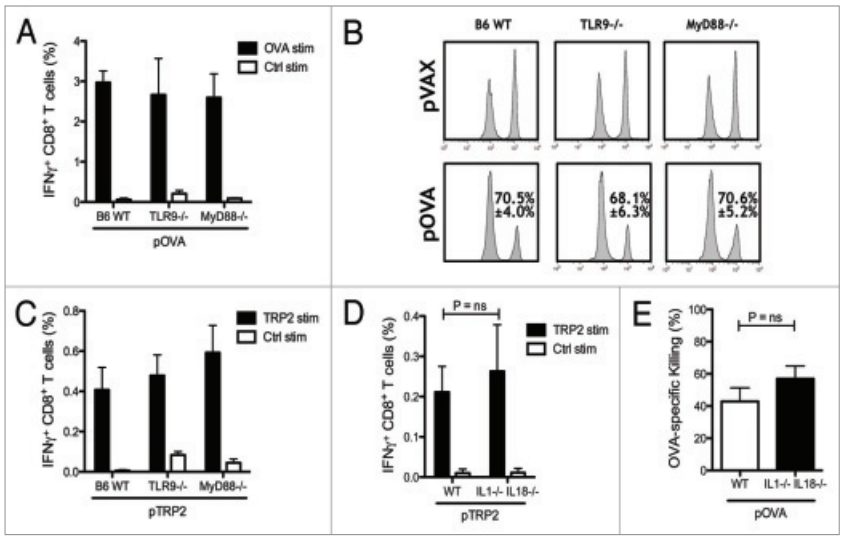

Figure 2. Toll-like receptor 9 (TLR9) and myeloid differentiation primary response gene 88 (MyD88) are not essential for CTL induction. Wild-type (WT), TLR9$^{-/-}$ and MyD88$^{-/-}$ mice on C57BL/6 background were vaccinated twice. Levels of IFN-γ producing CD8 T cells induced by DNA vaccination against OVA and TRP2 were measured two weeks after the second DNA vaccination by flow cytometry. Bars indicate the mean ± SEM (**A** and **C**). In vivo cytotoxicity was measured by quantifying OVA pulsed CFSE^{High} labeled target cells and compared with control pulsed CFSE^{Low} labeled cells in WT, TLR9$^{-/-}$ and MyD88$^{-/-}$ mice vaccinated with the OVA encoding plasmid pOVA. The mean percentage ± SEM of specific killing for each case is indicated (**B**). WT and IL-1β$^{-/-}$ IL-18$^{-/-}$ mice on C57BL/6 background were vaccinated with OVA or TRP2 encoded plasmid as described previously. The percentages of IFN-γ-producing CD8 T cells were measured by flow cytometry after in vitro stimulation with TRP2 and control peptides (**D**). Functionality of OVA-specific CTLs was measured by in vivo cytotoxicity. Lymph nodes were harvested one day after mice were injected i.v. with OVA peptide pulsed CFSE^{High} labeled splenocytes and control peptide pulsed CFSE^{Low} splenocytes. Bars indicate the mean percentage ± SEM of OVA-specific killing (**E**).

Discussion

The promise that DNA vaccines would lead to a revolution in vaccine technology has not yet materialized. Many clinical trials have been falling short of their mark with little explanation as to possible reasons, while in contrast, mouse models have shown therapeutic and prophylactic efficacy. In this work we attempt to shed some light on the mechanisms involved in establishing adaptive T cell responses elicited by DNA vaccination. We show here that naked plasmid DNA vaccines delivered via an intradermal route and assisted with electroporation produce antigen-specific CTL responses through a mechanism that relies on the activation of NF-κB. In contrast, the IRF3-type 1 IFN and TLR9-MyD88 innate immune pathways, generally considered to contribute to the sensing of DNA and the initiation of adaptive immune responses, were not required for establishing antigen-specific CTLs.

Electroporated DNA vaccines lead to high expression levels of the plasmid-encoded antigen as well as the introduction of large amounts of plasmid DNA inside the cells that represent a strong signal for several DNA-sensing innate immune receptors. The sensing of DNA by PRRs has become a complex research field, with a multitude of proteins binding to DNA and activating distinct signaling cascades, as recently reviewed in detail by Desmet et al.[21] The net result of these pathways is the production of type I IFNs and proinflammatory cytokines, including IL-1β and IL-18 that promote DC maturation and initiate robust cellular immune responses. In response to DNA, DAI, IFI16, DHX36, DDX41, RNA-PolIII, and LRRFIP1 trigger the production of type I IFNs primarily via TBK1-dependent activation of IRF3, whereas TLR9 and DHX9 can signal through MyD88 to activate IRF7. Type I IFNs lead to the maturation of DCs, inducing an increased expression of MHC molecules and costimulatory molecules, such as CD80 and CD86.[18] Importantly, type I IFNs also provide the "third signal" needed for CD8$^+$ T cells to undergo clonal proliferation and differentiation into CTLs.[17,25,26] The importance of the type I IFN signaling, in particular the TBK-1/IRF3-dependent pathway, for innate immune recognition of DNA vaccines has been established by studies showing that adaptive immune responses elicited by DNA vaccination are almost completely abrogated in mice lacking the components of this cascade.[19,20,27] Ishii et al. found that IFNαR$^{-/-}$ and Tbk1$^{-/-}$ mice were not able to generate LacZ specific CD8$^+$, as measured by MHC tetramers, or stimulate antigen-specific CD4$^+$ and CD8$^+$ T-cell proliferation.[20] Similarly, Shirota et al. found that IFNαR$^{-/-}$ and IRF3$^{-/-}$ mice displayed severely impaired antigen-specific CD4$^+$ and CD8$^+$ T cell responses, including the production of Th1, Th2, and Th17 cytokines.[19] In contrast to the aforementioned studies, in this study we present evidence demonstrating that, at least with DNA vaccines delivered by intradermal electroporation, IRF3- or MyD88-dependent type I IFN signaling is not required for the induction of fully functional antigen-specific T cell responses (**Figs. 1 and 2**). These opposing results can be explained by the different administration routes used in each particular case. Studies proposing a preponderant role for type I IFN signaling have been performed after intramuscular immunization

(with or without electroporation), whereas our studies were conducted using intradermal electroporation. It seems reasonable to expect that delivering DNA intramuscularly would target different sets of both DNA-sensing and antigen-presenting cells, than when injecting intradermally. Similar divergences have been found when immunizing with alphavirus replicon-based DNA vectors, which activate dsRNA-sensing innate immune receptors.[28,29] Induction of antigen-specific T cell responses elicited by vaccination with replicon-based DNA vectors delivered intramuscularly has shown to be dependent on type I IFNs[27] while in a different study immunizing with the same kind of vector but delivered intradermally, type I IFN signaling was absolutely dispensable, and rather had a suppressive effect.[30] The contrasting results obtained by different groups seem to be related to the vaccination route, further encouraging the need of more comprehensive studies dissecting the signaling pathways activated in each particular tissue.[30] Indeed, a different situation can be observed within the tumor microenvironment, where activation of type I IFN-producing PRRs has been shown to promote potent anti-tumor effects by acting on antigen-presenting cells and subsequently on tumor-specific T cells.[31,32] Treatment of B16F10 melanoma tumors with poly(I:C) and CpG oligos, triggering TLR3 and 9 respectively, protected mice from tumor challenge together with melanoma specific T-cell transfer. Poly(I:C) and CpG were shown to be responsible for upregulation of CD40 and CD86 on pDCs enriched from tumor draining lymph nodes.[31] In the context of vaccination, it has been found that intratumoral injection of TLR3/9 ligands was responsible for reprograming the infiltrating immune cell population; significantly increasing the CD8 to Treg cell ratio and anti-tumor effects of the vaccine strategy.[32]

In addition to type I IFNs, proinflammatory cytokines produced after DNA stimulation play a direct role in promoting DC maturation and T cell induction. Activation of TLR9-MyD88 pathway is a major source of both type I IFNs and proinflammatory cytokines. However, the participation of TLR9 in the immunogenicity of DNA vaccines is controversial with different studies showing TLR9-dependent and -independent mechanisms. In this study, we found that TLR9 and the adaptor protein MyD88 did not play an important role in establishing the pDNA induced adaptive CTL responses, which were similar between the KO and WT mice. In vivo cytotoxicity showed a trend to less effective killing in the KO mice, though this was not statistically significant (**Fig. 2**). Our findings are in agreement with previous investigations showing that TLR9 and MyD88 KO mice are able to mount antigen-specific CTL responses after i.m. DNA vaccination.[33,34] Considering all these studies, it can be concluded that TLR9 contribute but is dispensable for T cell responses elicited by DNA vaccines delivered, and targeting TLR9 represents an opportunity for improvement of adaptive immune responses. Indeed, strategies introducing more CpG motifs into the antigen-encoding plasmid DNA vector have been shown to further boost antibody titers in i.m. DNA vaccines.[35-37] A key proinflammatory pathway downstream of DNA recognition is the production of IL-1β and IL-18.[36] The pro-forms of the IL-1 family members are cleaved into their functional state by caspase-1 through interaction with AIM2-containing inflammasome. It has also been described by Roos et al. that IL-1β mRNA expression is increased 1000-fold after DNA electroporation in the skin.[39] Although AIM2 can be expressed in skin-resident cells, IL-1β$^{-/-}$ IL-18$^{-/-}$ mice were able to generate CTL responses as efficiently as wild-type mice further discarding that the inflammasome pathway is required for DNA vaccine-induced innate immune activation.

The maturation of DCs and the subsequent activation of adaptive immunity are regulated largely by NK-κB signaling pathways. The classical NF-κB signaling cascade triggered in response to dsDNA by DAI, IFI16, AIM2, DHX9, DDX41 DNA sensors,[21] relies on formation and activation of the NEMO/IκB kinase complex. This complex phosphorylates IκBα, facilitating the ubiquitination and proteasomal degradation of this protein. Once IκBα is degraded, it is no longer able to sequester NF-κB in the cytosol, the p50-RelA dimer then translocates to the nucleus.[40] Our studies show the key role of transcription factor NF-κB in establishing effective anti-tumor CTL responses by intradermal electroporation of plasmid DNA vaccines. By co-electroporating a mutant IκBα construct into the dermis of the skin along with antigen-encoding plasmids, the mechanism for triggering adaptive immunity is abrogated, preventing the tumor protection otherwise offered by DNA vaccines (**Fig. 3**). Electroporation of the IκBα-SR construct did not have an effect on tumor metastasis when administrated independently of the TRP2 encoding plasmid (data not shown). Among other targets, activation of NF-κB leads to the production of inflammatory cytokines, such as: IL-6, IL-1β, IL-12, and TNF-α. Among these molecules and similarly to type I IFNs, IL-12 is also able to provide the "third signal" to CD8$^+$ T cells for promoting clonal proliferation and differentiation into CTLs. Therefore, NF-κB can provide all the necessary signals for generating effective T cell immunity. Despite the ability of NF-κB to support malignant cell survival, proliferation, and metastatic potential when expressed in tumor cells;[41] activation of NF-κB during antigen presentation has been extensively demonstrated to promote anti-tumor immune responses using TLR agonists[27,28] and DNA vaccine adjuvants.[24] In conclusion, in our study we demonstrate the essential nature of NF-κB in establishing CTL immunity after intradermal DNA vaccination. However, identifying which of the multiple DNA-sensing receptors that is involved in sensing intradermally electroporated DNA will be the focus of future studies. A more complete understanding of the DNA-sensing signaling networks initiated after DNA vaccination will allow us to design more effective vaccines.

Materials and Methods

Animals and cells. C57BL/6, TLR9$^{-/-}$, MyD88$^{-/-}$, IRF3 $^{-/-}$, 129sv, and IFN-αR$^{-/-}$ mice were kept in accordance with the local Animal Ethics Committee guidelines. Mice were bred and maintained at the Microbiology and Tumor Biology center at the Karolinska Institute. B16F10 were cultured in complete RPMI medium, supplemented with 100 IU/ml penicillin and 100 μg/ml streptomycin, one percent non-essential amino acids, one percent sodium pyruvate and 10% heat-inactivated fetal bovine serum (Gibco 21875, 15140, 11140, 11360, and 10270 respectively) in a humidified incubator at 37°C with 5% CO_2.

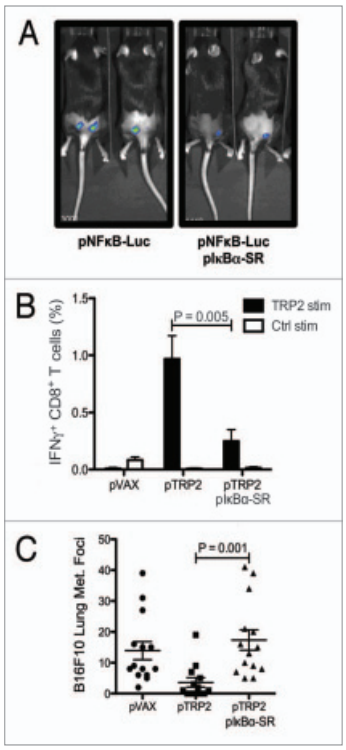

Figure 3. NF-κB activation during intradermal DNA vaccination mediates the generation of CTLs against the tumor-associated antigen TRP2. C57BL/6 mice were electroporated with NFκB luciferase reporter plasmid and IκBα-SR encoding plasmid. One day after DNA electroporation, in vivo luminescence was measured after intraperitoneal (i.p.) injection of luciferin. Representative images are shown (**A**). C57BL/6 mice were vaccinated twice against TRP2. One group was co-electroporated with IκBα-SR encoding plasmid and the control group with the empty vector. Percentage of TRP2-specific IFN-γ-producing CD8 T cells were measured two weeks after the second DNA vaccination by flow cytometry after in vitro stimulation with TRP2 and control peptides. Bars indicate the mean ± SEM (**B**). C57BL/6 mice vaccinated with pVAX, pTRP2 or co-electroporated with pTRP2 and pIκBα-SR were challenged by i.v. injection of 10^5 B16F10 melanoma cells. Two weeks after injection lungs were excised and melanoma foci were enumerated. The mean percentage ± SEM of melanoma foci is shown for each group (**C**).

DNA vaccination. Vaccination was done in mice as previously described.[16] Briefly, mice were anesthetized with isoflurane at gas concentration of 3.5%. Vaccination was delivered by injecting 20 μg of each plasmid DNA dissolved in 40 μl of phosphate-buffered saline intradermally. The intradermal injection was done in two different sites (20 μl each) on the hindquarters of the mouse. Electroporation of the injection site was done using the Derma Vax DNA Vaccine Skin Delivery System (Cellectis) that delivered electric pulses (two 1125 V/cm, 0.05 ms pulses followed by eight 275 V/cm, 10 ms pulses) via a parallel needle array electrode placed on the injection site. Immunization of the mice was done twice with a two-week interval. The plasmids used for immunization were as follows: pTRP2 encoding human TRP2 (kindly provided by Dr. T. Wölfel, Johannes Gutenberg University), pOVA encoding membrane-bound ovalbumin (kindly provided by Dr. A. Lew, Walter and Eliza Hall Institute of Medical Research), pIκBα-SR encoding the IκBα supperrepressor (kindly provided by Dr R. Toftgård, Karolinska Institutet), and pNF-κB-Fluc encoding firefly luciferase under the control of a NF-κB promoter. Plasmids were purified using EndoFree Plasmid Giga Kit (Qiagen 12391).

Intracellular cytokine staining. Peripheral blood was taken 13 d after the second DNA vaccination from the tail vein. Lymphocytes were cultured with 1 μg/ml of antigen-derived or control MHC class I-restricted peptide during eight hours. GolgiPlug was added during the last six hours. After surface staining, cells were stained intracellularly using the Cytofix/Cytoperm and fixation/permeabilization kit (BD Biosciences 554722 and 554723) following manufacturer's instructions. Monoclonal anti-mouse CD-8α FITC conjugated, IFN-γ PE conjugated TNF-α APC antibodies were used (Biolegend clones 5H10–1, XMG1.2 and MP6-XT22 respectively). Nonspecific binding was blocked by mouse Fc receptor blocking (Biolegend clone 93). Acquisition and analysis of cells was performed with FACSCalibur and FlowJo version 9.2, respectively.

In vivo cytotoxicity. Splenocytes harvested from naïve mice were pulsed with OVA or control peptide after being labeled with 2 and 0.2 μmol/l of CFSE respectively. Then, 10^7 pulsed splenocytes from each population were mixed and injected intravenously into vaccinated mice. The next day, inguinal lymph nodes from recipient mice were analyzed by flow cytometry. DNA vaccine mediated killing was determined as follows: 100 – (% of $CFSE^{high}$ OVA peptide-pulsed cells/% of $CFSE^{low}$ control peptide-pulsed cells).

In vivo luminescence. Six hours after C57BL/6 mice were vaccinated with plasmid encoding firefly luciferase, under control of an NF-κB promoter, luminescence was measured. This was done by anesthetizing the mice with isoflurane prior to intraperitoneally injecting 100 μl of 30 mg/ml D-luciferin (Promega E1603) dissolved in phosphate buffered saline solution. Mice were scanned with the Xenogen IVIS 100 imaging system where bioluminescence was measured.

Lung metastasis model. DNA vaccinated C57BL/6 mice were challenged by intravenous injection of 10^5 B16F10 cells 14 d after the second DNA vaccination. Three weeks after intravenous challenge, mice were sacrificed and lungs were washed in phosphate buffered saline solution before bleaching in Feketes solution. Analysis of metastasis was performed by enumeration of the visible pigmented melanoma foci in the lung surface.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

Research described here have been supported by grants to AL and RK from FONDECYT 11110525, CONICYT 791100038, CONICYT Program PFB-16, CORFO-Innova 12IDL2-13348, The Swedish Medical Research Council (K2011-66X-15387-07-3 VR), The Swedish Cancer Society (12 0598

Cancerfonden), The Cancer Society of Stockholm (121103 Cancerföreningen, Radiumhemmets Forskningsfonder), ALF-Project grant from Stockholm City Council (20110070 ALF Medicin 2012).

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III

DAI (DLM-1/ZBP1) as a Genetic Adjuvant for DNA Vaccines That Promotes Effective Antitumor CTL Immunity

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DNA vaccination is an attractive approach to induce antigen-specific cytotoxic CD8$^+$ T lymphocytes (CTLs), which can mediate protective antitumor immunity. The potency of DNA vaccines encoding weakly immunogenic tumor-associated antigens (TAAs) can be enhanced by codelivering gene-encoded adjuvants. Pattern recognition receptors (PRRs) that sense intracellular DNA could potentially be used to harness intrinsic immune-stimulating properties of plasmid DNA vaccines. Consequently, the cytosolic DNA sensor, DNA-dependent activator of interferon (IFN) regulatory factors (DAI), was used as a genetic adjuvant. *In vivo* electroporation (EP) of mice with a DAI-encoding plasmid (pDAI) promoted transcription of genes encoding type I IFNs, proinflammatory cytokines, and costimulatory molecules. Coimmunization with pDAI and antigen-encoding plasmids enhanced *in vivo* antigen-specific proliferation, and induction of effector and memory CTLs. Moreover, codelivery of pDAI effectively promoted CTL and CD4$^+$ Th1 responses to the TAA survivin. The DAI-enhanced CTL induction required nuclear factor κB (NF-κB) activation and type I IFN signaling, but did not involve the IFN regulatory factor 3 (IRF3). Codelivery of pDAI also increased CTL responses to the melanoma-associated antigen tyrosinase-related protein-2 (TRP2), enhanced tumor rejection and conferred long-term protection against B16 melanoma challenge. This study constitutes "proof-of-principle" validating the use of intracellular PRRs as genetic adjuvants to enhance DNA vaccine potency.

Received 20 July 2010; accepted 3 November 2010; published online 14 December 2010. doi:10.1038/mt.2010.268

INTRODUCTION

Cytotoxic CD8$^+$ T lymphocytes (CTLs) are key effector cells of the immune system and critical components of protective immunity against infectious diseases and cancer. Activated CTLs have the potential to eradicate malignant cells with high specificity. Indeed, intratumoral CTL infiltration is often associated with favorable clinical outcomes such as decreased disease recurrence and improved survival in diverse malignancies.[1–3] Adoptive transfer trials of tumor-specific CTLs that control disease progression in metastatic melanoma patients have provided direct evidence of the efficacy of CTLs.[4] Accordingly, induction of effective and long-lasting T cell immunity represents a major goal of cancer vaccines that have shown promising results in the clinic, especially when used as adjuvant therapy to standard cancer treatments in patients with minimal residual disease.[5]

DNA vaccination is an attractive and safe approach to generate protective CTL responses against cancer. This approach has been successful in animal models, but has shown limited efficacy in clinical trials.[6] One underlying reason is that most of the tumor-associated antigens (TAAs) recognized by T cells are normal nonmutated self-antigens and potentially self-reactive TAA-specific T cells are either eliminated or become regulatory T cells by mechanisms involving central and peripheral immune tolerance. Therefore, efficient delivery systems and potent adjuvants are needed for cancer DNA vaccines to overcome tumor-associated T cell tolerance. *In vivo* electroporation (EP) has emerged as a simple, efficient, and clinically applicable method for delivering DNA vaccines that greatly enhances plasmid uptake, antigen expression, and immune responses.[7] Moreover, DNA EP activates innate immunity resulting in infiltration of immune cells and the production of proinflammatory molecules that contribute to the induction of the immune responses.[8,9] The versatility of DNA vaccines facilitates codelivery of genes encoding immunomodulatory molecules, typically cytokines and chemokines, as genetic adjuvants. Moreover, the concerted action of several cytokines and costimulatory molecules clearly facilitates potent activation of the immune response.[10] One means to this end is the use of adjuvants, which exploit the immune-stimulating effects of pathogens by activating pattern recognition receptors (PRRs). PRRs are a group

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of evolutionary conserved innate immune receptors that sense pathogen-associated molecular patterns and activate downstream master transcription factors initiating the production of an array of cytokines, chemokines, and type I interferons (IFNs) to promote activation and maturation of adaptive immune responses.[11,12] A central feature of pattern recognition is the sensing of foreign nucleic acids. DNA-sensing PRRs include TLR9, located at endosomal compartments, and the recently described cytosolic sensors: DAI (also known as ZBP1 and DLM-1),[13] absent in melanoma 2 (AIM2),[14,15] and DNA-dependent RNA polymerase III.[16,17] *In vitro* studies revealed that DAI, the first cytoplasmic DNA sensor described, recognizes double-stranded DNA and triggers the gene expression of type I IFNs, IFN-inducible chemokine Cxcl10, and proinflammatory cytokines, such as interleukin 6 (IL-6) and tumor necrosis factor-α (TNF-α) via two distinct signaling pathways. One involves DAI-mediated phosphorylation of the TANK-binding kinase-1 and subsequent activation of the transcription factor IFN regulatory factor 3 (IRF3).[13,18] The other pathway requires phosphorylation of the receptor interacting protein-1 kinase, leading to phosphorylation of IκB-α, and subsequent activation of the transcription factor NF-κB.[19]

In the present study, we explored whether coexpressing a DNA-sensing PRR could further potentiate intrinsic adjuvant properties of bacterial plasmids used for DNA vaccines. Although DAI is dispensable for DNA vaccine immunogenicity,[20] we hypothesized that *in vivo* overexpression of DAI would boost DNA-induced innate immune activation and ultimately enhance adaptive T cell immunity.

RESULTS

DAI delivery promotes transcriptional upregulation of genes involved in innate and adaptive immunity *in vivo*

It has recently been established that intradermal (i.d.) DNA delivery followed by EP increases up to 200-fold the transcription of genes encoding proteins involved in immune regulation, such as chemokines, activation markers, and proinflammatory molecules.[21] To evaluate whether *in vivo* overexpression of DAI would further stimulate innate immunity, transcript levels of innate immune mediators were measured after DNA vaccination. Mice were inoculated i.d. with either control (pVAX) or DAI-encoding (pDAI) plasmids, followed by EP. Total RNA isolated from skin biopsies taken 24 hours later was analyzed by reverse transcription real-time PCR. Gene expression was normalized to the *L32* housekeeping gene. Although inoculation with pVAX or pDAI generated a similar local inflammatory response as determined by immunohistochemistry on electroporated skin sections (**Supplementary Figure S1 and Supplementary Materials and Methods**) and a similar upregulation of costimulatory molecules on dendritic cells isolated from the draining lymph node (**Supplementary Figure S2**), a significant increase ($P < 0.05$) was observed for genes encoding IFN-α, IL-6, TNF-α, and Cxcl10 in the pDAI-inoculated group relative to the control vector inoculated group (**Figure 1**). A trend toward increased IFN-β expression was also observed ($P = 0.055$). Interestingly, pDAI inoculation also led to significant upregulation of the genes encoding the costimulatory molecules CD40 and CD80 required for T cell activation and differentiation into effector T cells. Moreover, we found that i.d. EP with pDAI also upregulated transcription of genes involved in antigen presentation, T cell proliferation and maturation, growth factors, and antiviral responses as compared to control DNA using two real-time PCR arrays investigating the transcriptional regulation of >400 genes involved in the IFN and inflammatory responses (**Supplementary Table S1**). For instance, multiple genes encoding cytokines such as IL-5, -9, -13, -20, -21, -23, and -31 were upregulated. Interestingly, the most upregulated gene was *Ly75* (also DEC-205 or CD205), a C-type lectin receptor expressed on skin-resident dendritic cells involved in directing captured antigens to antigen-processing compartments.[22] Vaccines targeting antigen to DEC-205-expressing dendritic cells has been used as a strategy to enhance crosspresentation and T cell responses.[23] A list of the 20 most strongly upregulated genes, in addition to the genes assayed by the real-time PCR analysis, is provided in **Supplementary Table S1**.

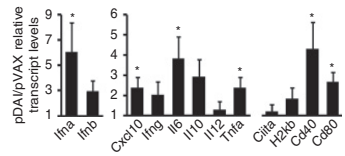

Figure 1 DAI EP promotes transcription of type I IFNs, proinflammatory cytokines, chemokine, and costimulatory molecules. C57BL/6 mice were electroporated with pVAX or pDAI ($n = 6$ per group) and skin biopsies were taken 24 hours later for gene expression analysis. Transcript levels of target genes were determined by quantitative real-time PCR. Relative pDAI/pVAX transcript levels represent the ratio between the levels detected in pDAI- and pVAX-injected mice. Bars are the mean ± SEM. *$P < 0.05$. Data presented are pooled from two independent experiments. EP, electroporation.

Codelivery of DAI promotes *in vivo* proliferation and induction of CTLs

Given the observed upregulation of proinflammatory and costimulatory transcripts in the skin, we studied whether pDAI coimmunization enhanced adaptive immunity. To test this, mice were covaccinated with pDAI and an ovalbumin (OVA)-encoding plasmid (pOVA), and compared with mice vaccinated with pOVA alone. To correct for the immunostimulatory effects exerted by plasmid DNA, the total quantity of DNA was adjusted by addition of a non-coding pVAX plasmid, so that the amount of DNA was the same in every animal. CFSE-labeled splenocytes from OT-I mice, whose $CD8^+$ T cells carry a transgenic TCR specific for the $OVA_{(257–264)}$ peptide, were adoptively transferred into the vaccinated mice. After 4 days, lymph nodes from recipient mice were isolated and the antigen-specific proliferation of OT-I $CD8^+$ T cells was analyzed by flow cytometry. Both division (**Figure 2a**) and proliferation (**Figure 2b**) indexes were calculated. These analyses show that in the pOVA+pDAI immunized mice, compared to the pOVA immunized mice, OVA-specific $CD8^+$ T cells divided significantly more (division indexes: 0.42 versus 0.26, $P = 0.02$; 0.14 is the basal level) and that dividing cells underwent more cycles of proliferation (proliferation indexes: 2.0 versus 1.6, $P = 0.007$; 1.23 is the basal level), indicating that codelivery of pDAI promoted a more efficient antigen presentation *in vivo*.

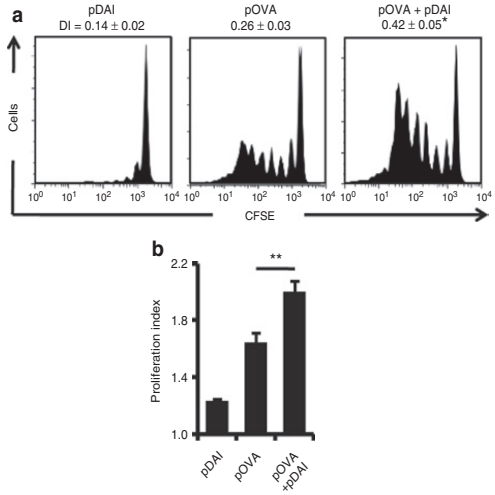

Figure 2 Codelivery of pDAI enhances antigen-specific proliferation of CD8$^+$ T cells. C57BL/6 mice were electroporated with pDAI, pOVA, or pOVA+pDAI (n = 6 per group). (**a**,**b**) *In vivo* antigen proliferation of adoptively transferred OT-I CD8$^+$ T cells was evaluated 4 days later. Representative histograms showing proliferation profiles of OT-I CFSE$^+$CD8$^+$Vα_2TCR$^+$ populations for each group are displayed including the (**a**) duplication index (DI) and the (**b**) proliferation index (PI) calculated for each group (mean ± SEM). *P = 0.02, **P = 0.007. Data shown are from one representative of two independent experiments.

The ability of DAI to induce functional OVA-specific CD8$^+$ T cells in peripheral blood following two immunizations was determined 13 days postimmunization by intracellular cytokine staining and flow cytometry analysis. Codelivery of pDAI increased the frequency of IFN-γ producing CD8$^+$ T cells from 2.5% observed in pOVA immunized mice to 4.4% (P = 0.01) (**Figure 3a**). Functional OVA-specific IFN-γ producing CD8$^+$ T cells could also produce TNF-α (**Figure 3b**). Using a H-2K^b:OVA$_{(257–264)}$ pentamer (**Supplementary Figure S3a**), we found that the OVA-specific CD8$^+$ T cells displayed a similar cytotoxic effector phenotype (CD25^{low}, CD69^{low}, CD44^{high}, CD62L^{low}; **Supplementary Figure S3b**) regardless of pDAI administration. To evaluate the cytotoxic potential of the antigen-specific CD8$^+$ T cells, spleen cells from naive mice were labeled with 0.2 or 2 µmol/l of CFSE and pulsed with control or OVA$_{(257–264)}$ peptides, respectively, and adoptively transferred to immunized mice. After 6 hours, lymph nodes from pDAI, pOVA, and pOVA+pDAI vaccinated mice were removed and the killing of the OVA$_{(257–264)}$-pulsed population (CFSE^{high}) relative to the control population (CFSE^{low}) was evaluated by flow cytometry. Vaccination with pOVA alone resulted in killing of 67% of the OVA$_{(257–264)}$-pulsed target cells. Coadministration of pDAI significantly (P = 0.017) increased the killing of the target population to 80% (**Figure 3c**), consistent with the observed increase in CTL frequency. No difference in antibody titers (**Supplementary Figure S4 and Supplementary Materials and Methods**) or immunoglobulin subclasses (data not shown) were observed between mice vaccinated with pOVA alone and pDAI+pOVA despite the induction of IFN-α (**Figure 1**) and cytokines such as IL-5 (**Supplementary Table S1**). In addition, the induction of different OVA$_{(257–264)}$-specific CD8$^+$ memory T cell subsets

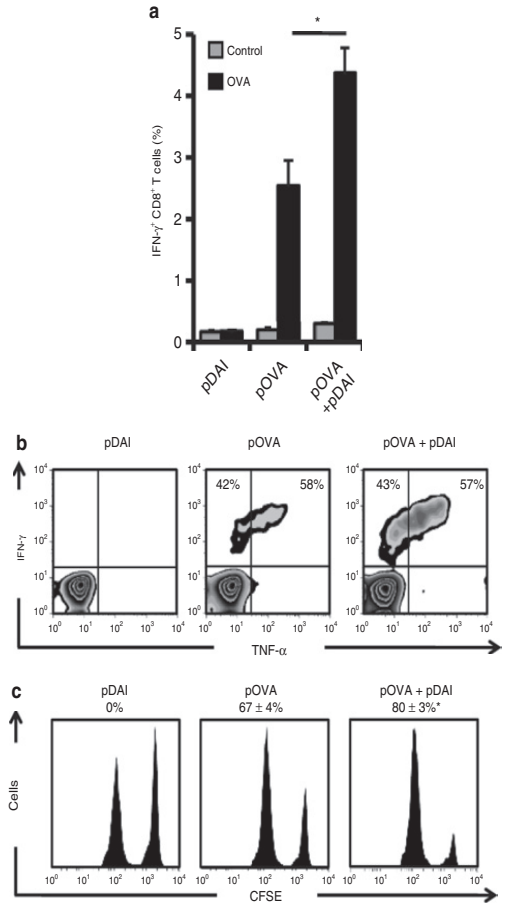

Figure 3 Codelivery of pDAI promotes antigen-specific cytotoxic T cell responses. C57BL/6 mice were electroporated with pDAI, pOVA, or pOVA+pDAI twice at 2-week interval. (**a**, **b**) CD8$^+$ T cell responses were analyzed in blood collected 13 days after the last vaccination (n = 6). (**a**) Frequency of IFN-γ producing CD8$^+$ T cells (over the gated CD8$^+$ T cell population) after *in vitro* stimulation with trp2$_{(180–188)}$ (control) or OVA$_{(257–264)}$ (OVA) peptides (**a**). Bars are the mean ± SEM. *P = 0.01. (**b**) Representative histograms showing IFN-γ and TNF-α expression on gated CD8$^+$ population. The relative proportion of IFN-γ$^+$TNF-α$^-$ and IFN-γ$^+$TNF-α$^+$ CD8$^+$ T cells is indicated. (**c**) *In vivo* cytotoxic killing of OVA$_{(257–264)}$-pulsed target cells (CFSE^{high}) relative to control trp2$_{(180–188)}$-pulsed cells (CFSE^{low}). A representative histogram per group is shown (n = 8) with the percentage of specific killing (mean ± SEM).*P = 0.017. Data shown are from one representative of at least two independent experiments. IFN, interferon; OVA, ovalbumin; TNF-α, tumor necrosis factor-α.

was determined by CD44 and CD62L staining (**Supplementary Figure S3c**) 5 weeks after the last vaccination. Both effector memory (T$_{EM}$; CD44^{high} CD62L^{low}) and central memory (T$_{CM}$; CD44^{high} CD62L^{high}) CD8$^+$ T cells were significantly increased (P = 0.024 and P = 0.031, respectively) in the spleen of mice coimmunized with pOVA+pDAI, whereas a similar trend was observed for CD8$^+$ memory stem cells (T$_{SCM}$; CD44^{low} CD62L^{high}) (**Figure 4a**). This analysis also showed that T$_{EM}$ and T$_{SCM}$ subsets were significantly increased in the blood (**Figure 4b**; P = 0.025 and P = 0.030,

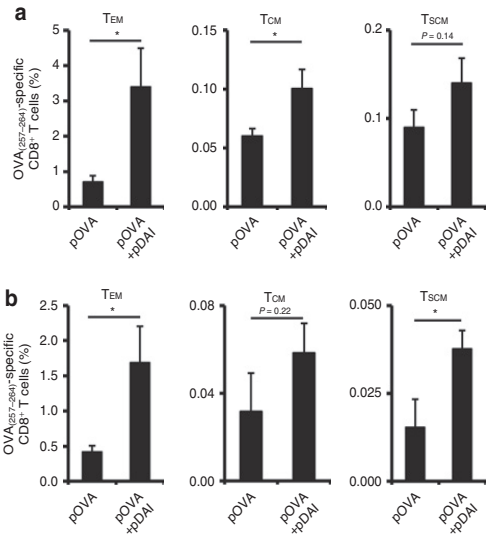

Figure 4 Codelivery of pDAI promotes memory CTL responses. C57BL/6 mice were electroporated with pDAI, pOVA, or pOVA+pDAI twice at 2-weeks interval ($n = 6$) and lymphocytes from (**a**) spleen and (**b**) blood were analyzed 5 weeks later by immunofluorescence staining and flow cytometry. Frequency of $OVA_{(257–264)}$-specific $CD8^+$ T cells (over the gated $CD8^+$ T cell population) showing one of the following memory subset phenotypes: effector memory (T_{EM}; $CD44^{high}CD62L^{low}$); central memory (T_{CM}; $CD44^{high}CD62L^{high}$); memory stem cells (T_{SCM}; $CD44^{low}CD62L^{high}$). Bars are the mean ± SEM. *$P < 0.05$. CTL, cytotoxic $CD8^+$ T lymphocytes.

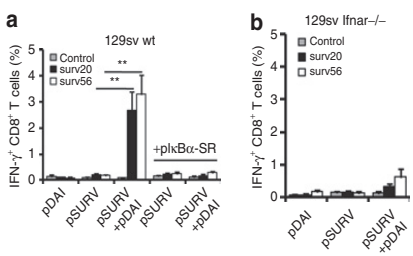

Figure 5 Codelivery of pDAI overcomes CTL tolerance to survivin TAA via a mechanism requiring NF-κB activation and type I IFN production. Mice were electroporated twice at 2-week intervals with pDAI, pSURV, or pSURV+pDAI ($n = 6$). Where indicated, pIkBα-SR was also administrated. (**a**, **b**) Peripheral lymphocytes were obtained from (**a**) 129Sv WT or (**b**) $Ifnar^{-/-}$ mice 13 days after the last vaccination. The frequency of IFN-γ producing $CD8^+$ T cells (over the gated $CD8^+$ T cell population) after *in vitro* stimulation with $trp2_{(180–188)}$ (control), $surv_{(20–28)}$ (surv20), or $surv_{(56–64)}$ (surv56) peptides is shown. Bars are the mean ± SEM. **$P = 0.002$. Data shown are from one representative of at least two independent experiments. CTL, cytotoxic $CD8^+$ T lymphocytes; IFN, interferon; NF-κB, nuclear factor κB; TAA, tumor-associated antigens; WT, wild type.

respectively). Altogether, this data shows that coadministration of pDAI effectively improves T cell immunity by enhancing *in vivo* antigen-specific proliferation of $CD8^+$ T cells and the induction and persistence of effector and memory CTLs.

DAI efficiently induces CTL immunity to survivin TAA and promotes tumor protection

We next studied whether DAI coadministration would enhance the immunogenicity of a weakly immunogenic TAA. Survivin is an intracellular inhibitor of apoptosis that is strongly upregulated in many types of cancer cells and associated with enhanced tumor cell viability.[24] While we were unable to detect responses to survivin in 129Sv mice vaccinated with a survivin-encoding plasmid (pSURV) alone, coadministration of pDAI overcame tolerance to the self-epitopes $surv_{(20–28)}$ and $surv_{(56–64)}$ and raised the frequency of IFN-γ-producing $CD8^+$ T cells in peripheral blood from non-detectable (<0.2%) to 2.7% ($P = 0.007$) and 3.3% ($P = 0.002$) for $surv_{(20–28)}$ and $surv_{(56–64)}$, respectively (**Figure 5a**). Given that most of the transcripts upregulated by DAI overexpression observed in **Figure 1** are potential targets for nuclear factor κB (NF-κB) (IFN-α, IL-6, TNF-α, Cxcl10, CD40, CD80),[25] we asked whether activation of this pathway was responsible for the adjuvant effect *in vivo*. A plasmid encoding a nonphosphorylable, degradation-resistant mutant of the NF-κB inhibitor, IκBα super-repressor (pIκBα-SR),[26] which blocks NF-κB-dependent transcription, was codelivered. Interestingly, coadministration of pIκBα-SR completely ablated the survivin-specific response observed in pDAI-vaccinated mice (**Figure 5a**), indicating that NF-κB activity is essential for the adjuvant effect of pDAI.

A hallmark of DAI signaling is the release of type I IFNs,[13,18] which are essential for optimal clonal expansion and enhanced effector function of CTLs.[27,28] Therefore, we investigated whether type I IFN production would contribute to the adjuvant effect of DAI. The induction of survivin-specific IFN-γ-producing CTLs observed in Sv129 mice vaccinated with pSURV+pDAI was drastically reduced in IFN-α/β receptor-deficient mice (**Figure 5b**). Codelivered pDAI could only generate an insignificant increase in the frequency of IFN-γ-producing $CD8^+$ T cells. This indicates that type I IFN production was largely responsible for the adjuvant effect of DAI, although other factors may also contribute.

DAI-induced type I IFN-secretion depends, at least partially, on IRF3-activation *in vitro*.[13] Therefore, we studied the effect of pDAI coadministration on survivin-specific CTL responses in IRF3-deficient mice. Coimmunization with pDAI+pSURV in both C57BL/6 wild type and $Irf3^{-/-}$ mice increased the frequency of antigen-specific IFN-γ-secreting $CD8^+$ T cells more than five-fold compared to pSURV alone, from 0.45 to 2.4% ($P < 0.0001$), or from 0.5 to 2.8–3.7% ($P < 0.005$), respectively (**Figure 6a,b**). These results indicate that IRF3 is not required for DAI-enhanced CTL induction. Interestingly, survivin-specific CTLs could simultaneously produce TNF-α and a small proportion also produced IL-2 (**Supplementary Figure S5**). We further tested whether pDAI coimmunization would promote $CD4^+$ Th1 responses to a major histocompatibility complex class II-restricted survivin epitope, $surv_{(53–67)}$. Indeed, pDAI increased the frequency of $CD4^+$ T cells producing simultaneously IFN-γ and TNF-α after $surv_{(53–67)}$ peptide stimulation from 0.049 to 0.19% (**Figure 6c**, $P < 0.05$). In order to assess its relative potency as a genetic adjuvant, pDAI was directly compared to the granulocyte-macrophage colony-stimulating factor (GM-CSF)-encoding plasmid (pGM-CSF), a widely used genetic adjuvant.[29] In contrast to pDAI, coimmunization with pSURV+pGM-CSF did not boost either $CD8^+$

or CD4$^+$ T cell responses to survivin (**Supplementary Figure S6**), indicating that DNA EP itself is a potent approach to elicit immune responses and probably masked the adjuvant effect of pGM-CSF observed by others including our group. Furthermore and considering the plethora of induced cytokines including IL-10, the potential induction of immunosuppressive cells such as CD25^{high}FoxP3$^+$ CD4$^+$ regulatory T cells, and Gr1$^+$CD11b$^+$ myeloid-derived suppressor cells was investigated in the inguinal lymph nodes and spleens from vaccinated mice 13 days postimmunization. Neither the vaccination with the TAA survivin nor the coimmunization with pDAI altered the frequency of regulatory T cells (**Supplementary Figure S7**). A small but significant ($P < 0.05$) decrease in myeloid-derived suppressor cells was detected in the spleen of mice coimmunized with pDAI+pSURV as compared to pSURV alone. The enhanced CTL and CD4$^+$ Th1 responses observed suggested that pDAI coadministration might enhance protection against tumor formation elicited by pSURV DNA vaccination. Indeed, administration of pDAI+pSURV to C57BL/6 mice demonstrated a trend ($P = 0.079$) toward improved rejection of a subcutaneous tumor challenge of the syngeneic B16 melanoma when compared to mice immunized with pSURV alone (**Figure 6d**).

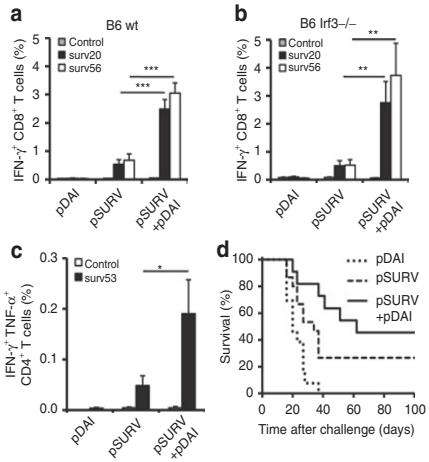

Figure 6 Codelivery of pDAI enhances CTL and CD4$^+$ Th1 responses to survivin TAA and promotes tumor protection. Mice were electroporated twice at 2-week intervals with pDAI, pSURV, or pSURV+pDAI. (**a**, **b**) Peripheral lymphocytes were obtained from (**a**) C57BL/6 WT or (**b**) Irf3$^{-/-}$ mice ($n = 8$) 13 days after the last vaccination. The frequency of IFN-γ producing CD8$^+$ T cells (over the gated CD8$^+$ T cell population) after *in vitro* stimulation with trp2$_{(180–188)}$ (control), surv$_{(20–28)}$ (surv20), or surv$_{(56–64)}$ (surv56) peptides is shown. Bars are the mean ± SEM. ***$P <$ 0.0001, **$P = 0.005$. Data shown are from one representative of at least two independent experiments. (**c**) Peripheral lymphocytes were obtained 13 days after the last vaccination ($n = 7$). The frequency of peripheral CD4$^+$ T cells producing simultaneously IFN-γ and TNF-α (over the gated CD4$^+$ T cell population) after *in vitro* stimulation with ova$_{(323–339)}$ (control) or surv$_{(53–67)}$ (surv53) peptides is shown. Bars are the mean ± SEM. *$P <$ 0.05. (**d**) Survival of C57BL/6 WT mice challenged with B16 melanoma cells 2 weeks after the second vaccination (pDAI, $n = 5$; pSURV, $n = 15$; pSURV+pDAI, $n = 11$). $P = 0.079$ for the comparison between pSURV and pSURV+pDAI groups. Data presented were pooled from two independent experiments. CTL, cytotoxic CD8$^+$ T lymphocytes; IFN, interferon; TAA, tumor-associated antigens; WT, wild type.

DAI enhances TRP2-specific CTL responses and confers long-term tumor protection

In order to confirm the ability of DAI to enhance antitumor CTL responses, we combined pDAI with a tyrosinase-related protein-2 (TRP2) encoding DNA vaccine (pTRP2). TRP2 is a highly expressed glycoprotein in human melanomas and a clinically relevant model antigen used for CTL-mediated targeting of mouse B16 melanoma.[30] An increased frequency of trp2$_{(180–188)}$-specific IFN-γ-producing CD8$^+$ T cells was observed in mice covaccinated with pTRP2 and pDAI (pTRP2+pDAI) as compared to mice vaccinated with pTRP2 only (0.75 and 0.43% respectively, $P = 0.043$) (**Figure 7a**). We also tested whether pDAI covaccination would increase the rejection of B16 melanoma challenge and found that a higher proportion of the covaccinated mice rejected a B16 melanoma challenge compared to mice vaccinated with TRP2 alone (**Figure 7b**; $P = 0.044$). Given the enhancement of antigen-specific memory CD8$^+$ T cell responses observed in **Figure 4**, mice that survived the B16 challenge from both pTRP2 and pTRP2+pDAI vaccinated groups were rechallenged 3 months after the initial challenge with a higher dose

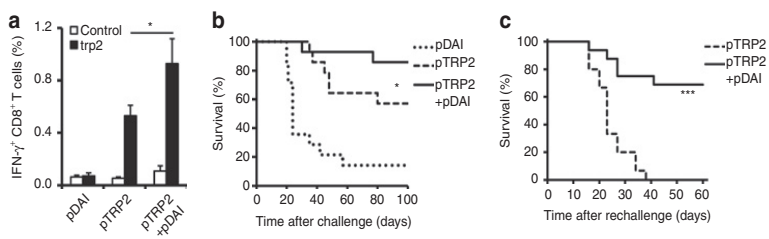

Figure 7 Codelivery of pDAI enhances TRP2-specific CTL responses and confers long-term protection to B16 melanoma challenge. C57BL/6 mice were electroporated twice at 2-week intervals with pDAI, pTRP2, or pTRP2+pDAI. (**a**) Peripheral lymphocytes were obtained 13 days after the last vaccination. The frequency of IFN-γ producing CD8$^+$ T cells (over the gated CD8$^+$ T cell population) after *in vitro* stimulation with surv$_{(20–28)}$ (control) or trp2$_{(180–188)}$ (trp2) peptides is shown ($n = 6$). Bars are the mean ± SEM. *$P = 0.043$. Data presented are from one representative of two independent experiments. (**b**) Survival of C57BL/6 WT mice challenged with B16 melanoma cells two weeks after the second vaccination ($n = 14$). *$P = 0.044$ for the comparison between pTRP2 and pTRP2+pDAI groups. Data presented were pooled from two independent experiments. (**c**) Long-term protection of C57BL/6 WT mice that had rejected the initial tumor challenge and were rechallenged with a higher dose of B16 melanoma cells in the opposite flank 3 months after initial challenge (pTRP2, $n = 15$; pTRP2+pDAI, $n = 16$). Data presented were pooled from two independent experiments. ***$P < 0.0001$. CTL, cytotoxic CD8$^+$ T lymphocytes; IFN, interferon; TRP2, tyrosinase-related protein-2; TAA, tumor-associated antigens; WT, wild type.

of B16 melanoma cells, without any additional immunization. Remarkably, almost 70% of the pTRP2+pDAI covaccinated mice rejected the melanoma cells whereas all mice vaccinated with pTRP2 alone succumbed to the second challenge ($P < 0.0001$) (**Figure 7c**). These results argue that DAI not only promotes the induction of functional effector cells, but also enhances immunological memory.

DISCUSSION

In the present study, we describe for the first time the use of an intracellular PRR as a genetic adjuvant that enhances the immunogenicity of DNA vaccines. DNA vaccines encode single (or few) antigens, which makes them highly specific but also inherently less immunogenic than whole-cell or multicomponent vaccines developed in the past. Furthermore, DNA vaccines against cancer need to overcome the tumor-associated immune tolerance, which is generally characterized by low frequencies of TAA-specific T cell precursors or the presence of TAA-specific T cells with intermediate-low TCR affinity. Therefore, adjuvants that enhance the immunogenicity of the encoded antigen and efficiently induce TAA-specific effector T cells are essential components of antitumor DNA vaccines. Genes encoding single cytokines and chemokines have been extensively used as genetic adjuvants. Notably genes encoding *IL-2*, *IL-12*, *IL-15*, and *GM-CSF* have successfully adjuvanted DNA vaccines in mice and nonhuman primates[31–33] as well as in humans.[34] However, some of these molecules, *e.g.*, IL-2[35] and GM-CSF,[36,37] are important in maintaining immune tolerance to self-antigens. Moreover, recent results from clinical studies have shown that IL-2 or GM-CSF as adjuvant for cancer vaccines or immunotherapies can promote the induction and recruitment of immunosuppressive T regulatory cells[38,39] and myeloid-derived suppressor cells.[40] The homeostatic roles of certain cytokines and sometimes the contradictory effects observed in clinical trials have raised some concerns about the use of single cytokines as adjuvants for cancer vaccines. Strategies involving the concerted action of several cytokines and costimulatory molecules can result in a potent activation of the immune responses, in particular against weakly immunogenic antigens.[10] The production of several immunostimulatory molecules can be achieved by stimulation of innate immune PRRs, as occurs during natural infection. Consequently, strategies that boost innate immune PRR signaling by coexpressing intracellular adaptor molecules or downstream transcription factors as genetic adjuvants, have been shown to enhance the potency of DNA vaccines.[41,42] However, such strategies have not been combined with antitumor DNA vaccines. The strategy developed in this study consists of delivering both the DNA-encoded intracellular PRR (DAI) and its activating ligand (plasmid DNA) to stimulate downstream transcription factors and initiate the production of several proinflammatory and costimulatory molecules, as well as type I IFNs, which ultimately promote adaptive T cell responses. The coadministration of pDAI was indeed comparatively more potent than the plasmid encoding GM-CSF in enhancing survivin-specific T cell responses in the experimental setup described here (**Supplementary Figure S6**). Moreover, pDAI did not induce immunosuppressive cell populations, as it has been reported for other adjuvants,[38–40] and rather showed decreased levels of myeloid-derived suppressor cells (**Supplementary Figure S7**).

In vitro studies have demonstrated that DAI signaling can activate both NF-κB and IRF3 to produce type I IFNs and other proinflammatory cytokines.[13,18] Nevertheless, little is known about DAI signaling *in vivo*. Our data show that i.d. EP with pDAI induces transcriptional upregulation of molecules that are known downstream targets of NF-κB (**Figure 1**), indicating that this key mediator of the innate immunity was activated in mouse skin. NF-κB represents a master transcription factor for signaling through TLRs[43] and intracellular DNA-sensing PRRs.[13,15,16,19] Consistent with such a crucial role in DNA-induced innate immune activation, NF-κB was essential for DAI-promoted CTL induction (**Figure 4a**) and, quite unexpectedly, this effect was not dependent on IRF3 (**Figure 5b**). Based on these observations, the critical role of type I IFNs for DAI-enhanced CTL induction (**Figure 4b**) and that IRFs are the main transcription factors driving type I IFN expression, we speculate that NF-κB activation is required for the initial release of type I IFNs and the resulting signaling events then activate IRFs, other than IRF3, to ensure robust induction of innate immune responses. Type I IFN production has been described to occur in two waves (reviewed in ref. 44). In the first wave, PRR signaling results in activation of NF-κB, IRF3, or both, and subsequent release of IFN-β. In the second wave, IFN-β transmits the danger signal to neighboring cells through binding to the type I IFN receptor. This turns on a signaling cascade that promotes gene expression of IFN-inducible genes with antiviral activity as well as IRF7. Possibly, IRF7-signaling contributes to ensure type-I IFN responses in the absence of IRF3-signaling,[13] thus sufficing the production of large amounts of type I IFNs.

The effects of type I IFNs include the release of cytokines and chemokines that modulate the function of dendritic cells resulting in, among other things, increased major histocompatibility complex class I crosspresentation, as well as improved development of effector and memory CTLs.[45,46] Our results support the notion that, in addition to antigen and coreceptor mediated stimulation, a third cytokine signal is important for effective CTL induction.[27,28] Among the cytokines that can provide the third signal, IFN-α but not IL-12 transcripts were upregulated after pDAI EP (**Figure 1**). Also, type I IFN signaling was essential for DAI-mediated CTL induction (**Figure 4b**). There is abundant evidence supporting the importance of type I IFNs in the generation of long-lasting antitumor immunity. Type I IFNs enhance tumor protection by increasing induction, proliferation, effector function, resistance to apoptosis, and long-term effector memory phenotype of CTLs.[47] On the other hand, defective type I IFN signaling and downstream activation of T cells is a common immune dysfunction in patients with different types of cancer.[48] Here, we demonstrate that pDAI coimmunization promotes the induction and persistence of memory $CD8^+$ T cell of effector, central, and stem cell phenotypes (**Figure 4**). Accordingly, the combination of DAI- and TRP2-encoding plasmids elicited long-term protection against B16 melanoma (**Figure 6c**).

In summary, our studies show that *in vivo* overexpression of DAI boosts DNA-sensing innate immune activation and thereby generates a proinflammatory microenvironment essential for

effective CTL induction and long-lasting antitumor immunity. Thus, this study validates the use of intracellular innate PRRs as genetic adjuvants that harness intrinsic innate immune-stimulating properties of plasmid DNA vaccines to enhance the immunogenicity of weakly immunogenic antigens. Hence, our findings are expected to improve the design of DNA vaccines for diseases where efficient cellular immunity is desired to confer protection.

MATERIALS AND METHODS

Mice and immunizations. C57BL/6, OT-I Rag-1$^{-/-}$, Irf3$^{-/-}$, and 129Sv and Ifnar$^{-/-}$ mice were kept according to the guidelines of the Regional Animal Ethics Committee. Mice anesthetized with isoflurane were injected i.d. with 40 µl of phosphate-buffered saline containing 20 µg of each plasmid at two different sites (20 µl each). pVAX was used to equalize DNA quantity within the same experiment. A parallel needle array electrode (two rows of four 2-mm pins, 1.5 × 4 mm gaps) was applied to deliver the electric pulses (two 1,125 V/cm, 0.05 ms pulses followed by eight 275 V/cm, 10 ms pulses)[49] using the Derma Vax DNA Vaccine Skin Delivery System (Cyto Pulse Sciences, now Cellectics, Romainville, France). Mice were immunized two times with 2 weeks between immunizations. The following plasmids were used for immunizations: pOVA encoding membrane-bound OVA (kindly provided by Dr A. Lew, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia), pSURV encoding the human survivin gene has been described,[50] pTRP2 encoding the human TRP2 (kindly provided by Dr T. Wölfel, Johannes Gutenberg University, Gutenberg, Germany), pIκBα-SR encoding the IκBα super-repressor (kindly provided by Dr R. Toftgård, Karolinska Institutet, Stockholm, Sweden) and pDAI. pDAI was produced by cloning the DAI coding sequence from mouse splenocytes into the pVAX vector (Invitrogen, Carlsbad, CA) using standard cloning procedures. Primers used for DAI cloning are listed in **Supplementary Table S1**. Plasmids were purified using the GigaPrep Endofree Kit (Qiagen, Hilden, Germany). Tumor challenge and rechallenge was performed by injecting subcutaneously 1×10^5 and 2×10^5 B16 cells, respectively.

Quantitative real-time reverse transcription-PCR. Total RNA was isolated from skin biopsies taken 24 hours after DNA EP and cDNA was prepared (iScript cDNA synthesis kit; Bio-Rad, Hercules, CA). Transcript levels were determined by quantitative real-time PCR (iQ SYBR Green Supermix; Bio-Rad; ABI7500, Applied Biosystems, Foster City, CA) using a two-step cycling program (1 minute at 95 °C, followed by 40 cycles of 15 seconds at 95 °C and 1 minute at 62–64 °C) and normalized to the *L32* housekeeping gene. Primers are listed in **Supplementary Table S2**. Pooled cDNA from the pVAX and pDAI groups was added to the RT^2 SYBR Green qPCR Master Mix (SABiosciences, Frederick, MD) and each sample was aliquoted on the Mouse IFN-α, β response, and the mouse inflammatory response and autoimmunity RT^{2}Profiler PCR-arrays, respectively. All steps were done according to the manufacturer's protocol for the ABI Prism 7500 and 7900 HT Sequence Detection System. To analyze the PCR-array data, excel macros were downloaded from the manufacturer's website (http://www.sabiosciences.com/pcrarraydataanalysis.php). Data normalization was based on correcting all C_t values for the average C_t values of several constantly expressed housekeeping genes present on the array.

Antibodies and flow cytometry. Monoclonal antibodies anti-mouse CD8α (clone 53-6.7), IFN-γ (clone XMG1.29), TNF-α (clone MP6-XT22), Vα2 TCR (clone B20.1), IL-2 (clone JES6-5H4), CD11b (clone M1/70), CD11c (clone N418), CD25 (clone PC61), CD40 (clone 1C10), CD44 (clone IM7), CD62L (clone MEL-14), CD69 (clone H1.2F3), CD80 (clone 16-10A1), FOXP3 (MF23), major histocompatibility complex class II (clone M5/114.15.2), Gr1 (RB6-85C) (BD Biosciences, San Jose, CA) and H-2Kb/OVA$_{(257–264)}$ pentamer (Proimmune, Oxford, UK) were used for immunoflorescence staining and flow cytometry analysis. Nonspecific binding was blocked by adding unconjugated rat anti-mouse CD16/CD32 antibody (mouse BD Fc block, clone 2.4G2; BD Biosciences). Samples were analyzed on a FACSCalibur cytometer (BD Biosciences) and the data were processed using FlowJo version 6.4.7 (Tree Star, Ashland, OR).

Intracellular cytokine staining. Peripheral blood was collected 13 days after the last immunization and lymphocytes were stimulated with peptides (1 µg/ml) for 8 hours. GolgiPlug (BD Biosciences) was added after 2 hours. Intracellular staining was performed using Cytofix/Cytoperm Fixation/Permeabilization Solution set (BD Biosciences) according to the manufacturer's instructions.

In vivo *antigen proliferation of OT-I CD8+ T cells.* Spleen and lymph node cells from naive OT-I mice were isolated. Splenocytes were RBC depleted using PharmLyse buffer. Cells (10^8 cells/ml; phosphate-buffered saline 0.5% RPMI) were stained with 2 µmol/l CFSE (Sigma-Aldrich, St Louis, MO) for 5 minutes. A tenfold larger volume of 20% fetal bovine serum RPMI was added to stop CFSE staining. Cells were then washed twice, resuspended in phosphate-buffered saline at a final concentration of 10^7 cells/ml and 2×10^6 cells were intravenously transferred into vaccinated recipients. Inguinal lymph nodes were sampled after 4 days and analyzed by flow cytometry.

In vivo *cytotoxicity assay.* Target spleen cells were labeled with 0.2 and 2 µmol/l of CFSE, and pulsed with control or OVA peptide, respectively. Then, 10^7 cells from each population were mixed and injected intravenously into vaccinated mice. Lymph nodes were removed 6 hours later and analyzed by flow cytometry.[50] CFSE$^+$ donor target splenocytes were differentiated from host cells and the percentage of specific killing was determined as follows: 100 – [(% of OVA peptide-pulsed targets/% of TRP2 peptide-pulsed targets in vaccinated recipients)/(% of OVA peptide-pulsed targets/% of TRP2 peptide-pulsed targets in control vaccinated recipients) × 100].

Statistical analysis. Statistical analysis was performed using the Graphpad Prism software (Graphpad Software, La Jolla, CA). Unpaired *t* tests were performed pair wise between relevant groups. No multiple comparisons were performed to control for type I errors. Statistical analyses of survival curves were performed using the one-tailed Mantel–Cox log-rank test.

SUPPLEMENTARY MATERIAL

Figure S1 . Intradermal DNA electroporation induces tissue inflammation.
Figure S2. DNA vaccine-induced upregulation of costimulatory molecules in dendritic cells from skin-draining lymph nodes.
Figure S3. Phenotypic analysis of DNA vaccine-induced OVA-specific CTLs.
Figure S4. Detection of DNA vaccine-induced OVA-specific antibodies.
Figure S5. DNA vaccine-induced survivin-specific CTLs produced IFN-γ, TNF-α, and IL-2 after peptide stimulation.
Figure S6. Comparison of the adjuvant efficacy of pDAI and pGM-CSF to enhance survivin-specific CTL and Th1 responses.
Figure S7. Analysis of immunosuppressive cell populations.
Table S1. The 20 most strongly upregulated gene transcripts in mice electroporated with pDAI as compared to mice electroporated with pVAX control vector.
Table S2. Primers used for cloning and quantitative real-time PCR analysis.
Materials and Methods.

ACKNOWLEDGMENTS

This work was supported by grants to R.K. from the Swedish Cancer Society, the Swedish Medical Research Council, the Cancer Society of Stockholm, the European Union (Grant "EUCAAD" and "DC-THERA"), the Karolinska Institutet, "ALF-Project" grants from the Stockholm

City Council. AFGQ has received support from ICGEB (International Center of Genetic Engineering and Biotechnology, Trieste, Italy) grant CRP/CH102-01, Wellcome Trust award WT06491I/Z/01/Z and FONDAP grant 15010006. A.L. has been supported by a Fellowship for Postgraduate Studies "Presidente de la República" from CONICYT, Chile. D.M. was supported by a grant of the German Research Association (DFG). K.L. has been supported by a postdoctoral fellowship from the Swedish Society for Medical Research.

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Figure S1
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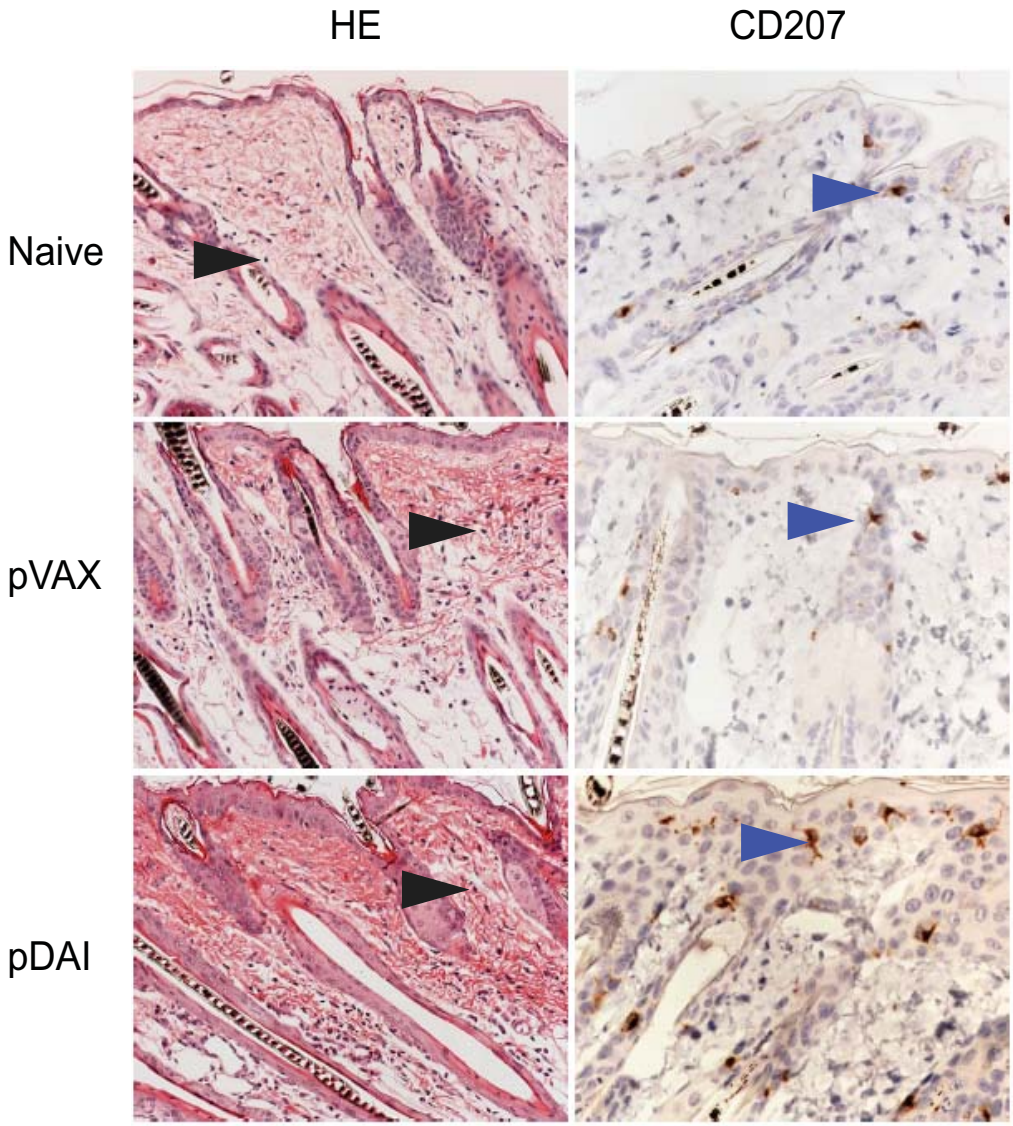

Figure S2 Intradermal DNA electroporation induces tissue inflammation. Mice were vaccinated and biopsies were collected as in Figure 1. H&E staining reveals massive infiltration of lymphocytes (black cells, black arrowheads) in DNA electroporated skin (left panel). Significant skin infiltration by $CD207^+$ DCs (brown, blue arrowheads) is observed in pVAX and pDAI electroporated mice when compared to untreated controls ($p=0.007$ and 0.0004, respectively) as detected by anti-CD207 staining (right panel).

Fig S2
Lladser *et al.*

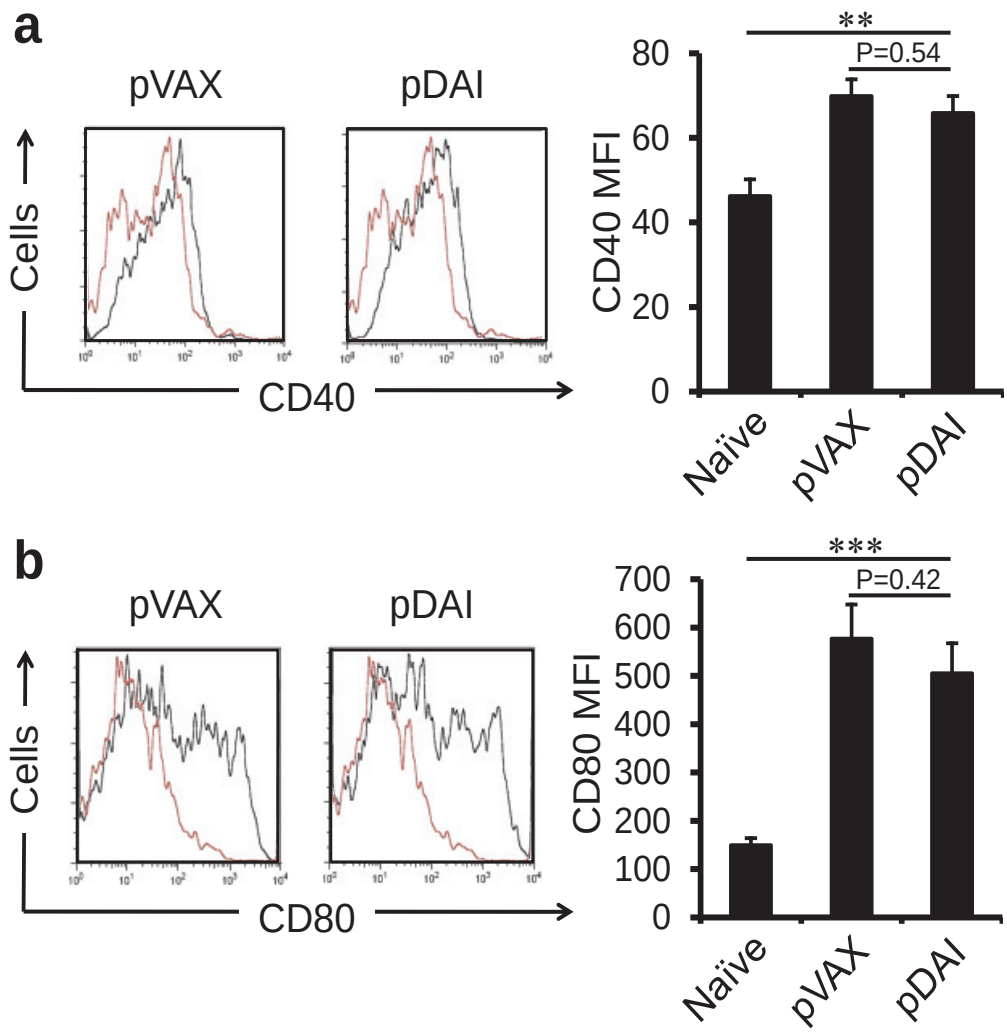

Figure S2 DNA vaccine-induced upregulation of costimulatory molecules in dendritic cells from skin-draining lymph nodes. C57BL/6 mice (n=6) were vaccinated as in Figure 1 and the inguinal lymph nodes were taken 24 h later. The levels of costimulatory molecules CD40 and CD80 were analyzed in dendritic cells ($CD11c^+$, MHC class II^{high}) by surface staining (black histograms) using naïve mice as controls (red histograms) and the mean fluorescence intensity (MFI) was calculated for each group. Bars are the mean±SEM. **indicates p=0.007; ***indicates p=0.0001.

Fig S3
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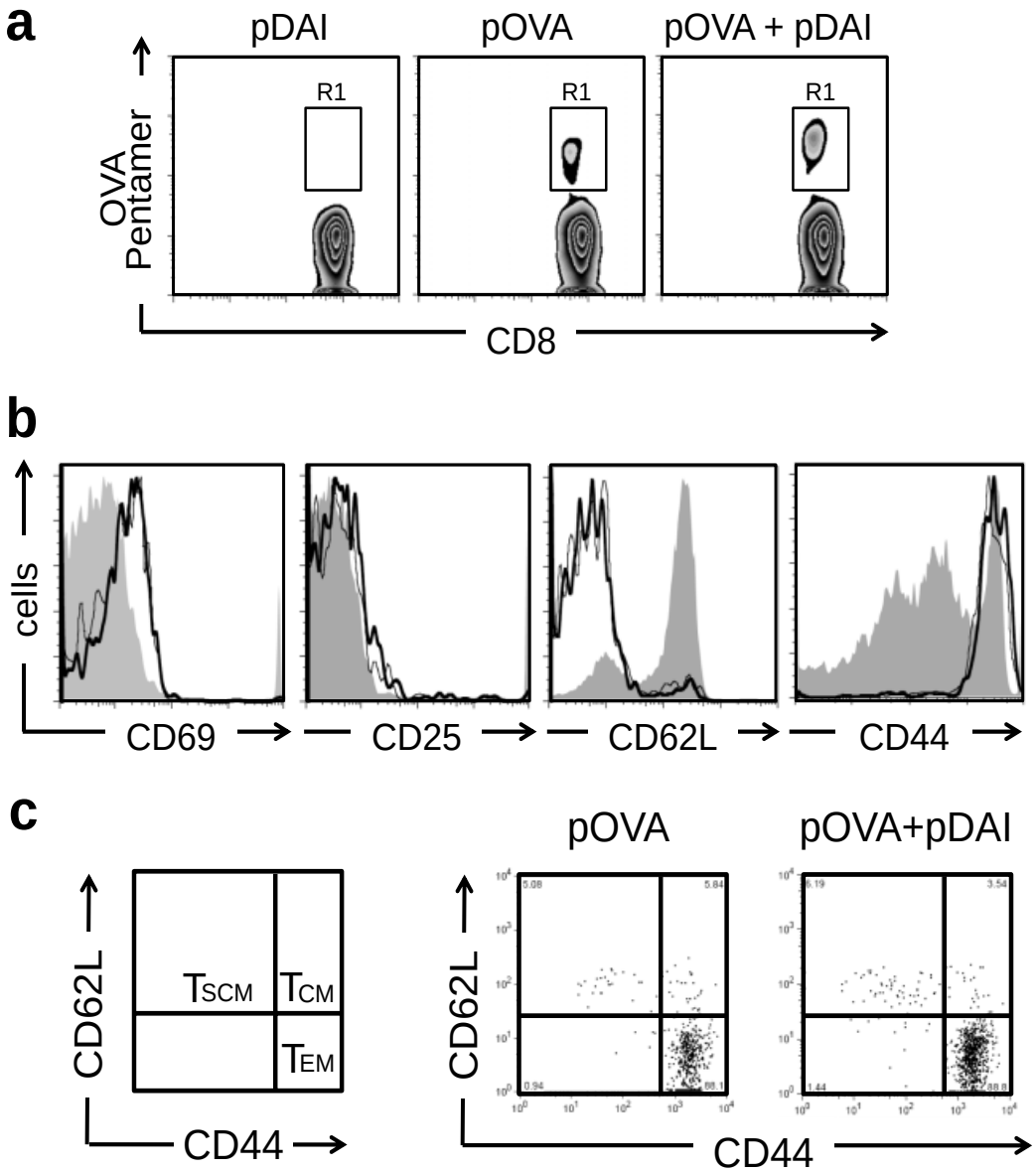

Figure S3 Phenotypic analysis of DNA vaccine-induced OVA-specific CTLs. C57BL/6 mice were vaccinated twice as in Figure 3 (n=6). (**a**) Detection of $OVA_{(257-264)}$-specific $CD8^+$ T cells (R1) was performed by pentamer staining either at two (**b**) or five (**c**) weeks after the last vaccination. (**b**) Surface staining of phenotypic markers on $OVA_{(257-264)}$-specific $CD8^+$ T cells was evaluated in pooled peripheral blood from mice immunized with pOVA (—, thin line profiles) or pOVA+pDAI (—, thick line profiles). The bulk $CD8^+$ T cell population (▪, solid grey profiles) was used as control. (**c**) Analysis of $OVA_{(257-264)}$-specific CD8+ T cells to identify the different memory sub-set phenotypes: effector memory (T_{EM}; $CD44^{high}CD62L^{low}$); central memory (T_{CM}; $CD44^{high}CD62L^{high}$); memory stem cells (T_{SCM}; $CD44^{low}CD62L^{high}$).

Figure S4
Lladser *et al.*

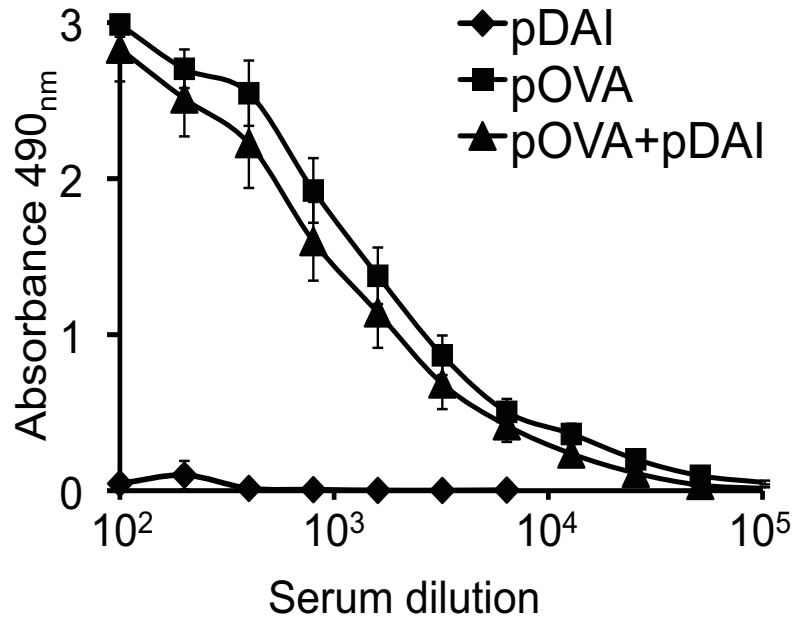

Figure S4 Detection of DNA vaccine-induced OVA-specific antibodies. C57BL/6 mice were vaccinated twice as in Figure 3 and sera collected 13 days after the last vaccination. OVA-specific antibodies were detected by indirect ELISA in sera from mice immunized with pDAI (♦, diamonds), pOVA (▪, squares) or pOVA+pDAI (▲, triangles) (mean±SEM; n=8). No statistically significant differences in antibody levels were observed between pOVA and pOVA+pDAI groups.

Fig S5
Lladser *et al.*

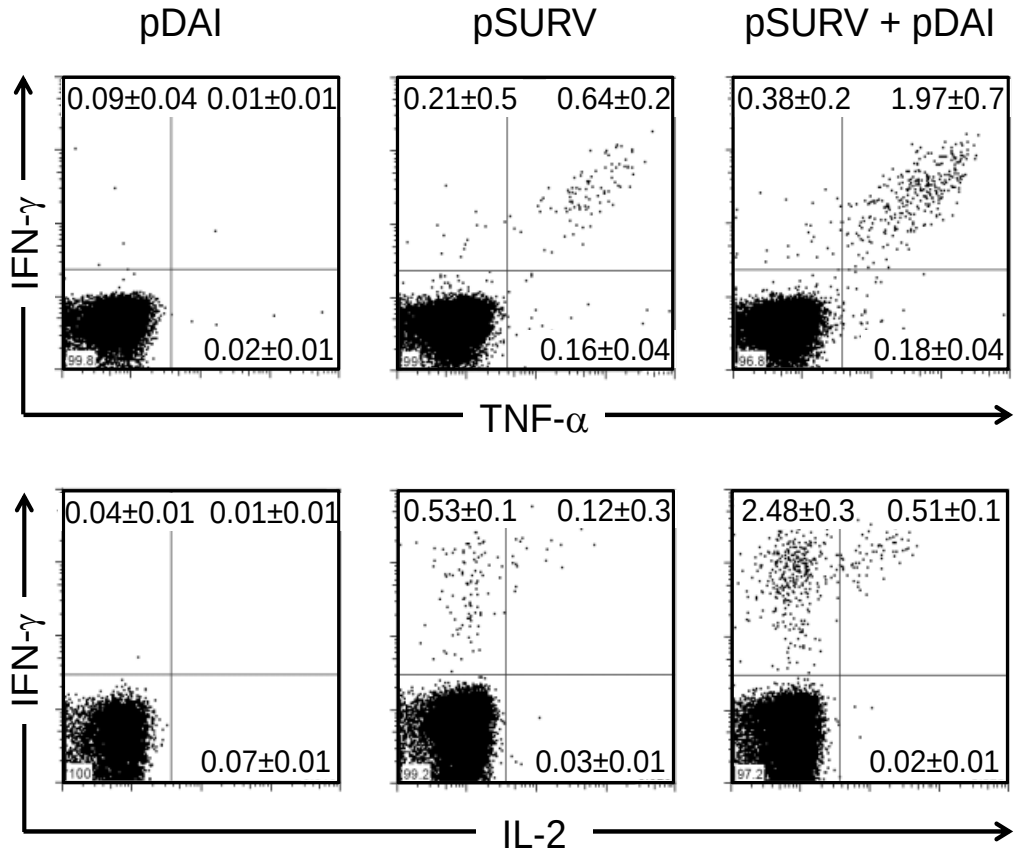

Figure S5 DNA vaccine-induced survivin-specific CTLs produced IFN-γ, TNF-α, and IL-2 after peptide stimulation. C57BL/6 mice were vaccinated as in Figure 5 and blood collected 13 days after the last vaccination. Detection of survivin-specific $CD8^+$ T cells was performed after in vitro stimulation with $surv_{(56-64)}$ peptide. Intracellular staining of IFN-γ simultaneously with TNF-a or IL-2 in gated $CD8^+$ T cells from mice immunized with pDAI (left panels), pSURV (middle panels) or pSURV+pDAI (right panels). Dot plots from a representative mouse per group are displayed indicating the mean±SEM for each group (n=4). Similar results were obtained after stimulation with $surv_{(20-28)}$ peptides.

Fig S6
Lladser *et al.*

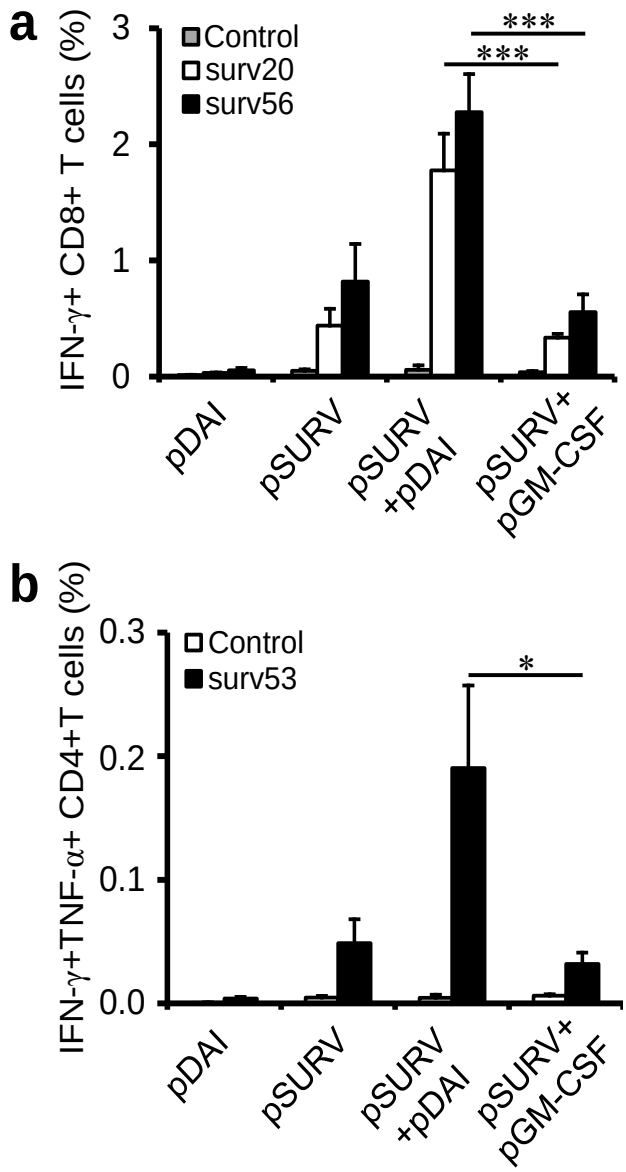

Figure S6 Comparison of the adjuvant efficacy of pDAI and pGM-CSF to enhance survivin-specific CTL and Th1 responses. C57BL/6 mice were electroporated twice at two-week intervals with pDAI, pSURV, pSURV+pDAI or pSURV+pGM-CSF (n=7) and blood collected 13 days after the last vaccination. (**a**) The frequency of peripheral IFN-γ-producing $CD8^+$ T cells (over the gated $CD8^+$ T cell population) after *in vitro* stimulation with $trp2_{(180-188)}$ (Control), $surv_{(20-28)}$ (surv20) or $surv_{(56-64)}$ (surv56) peptides is shown. (**b**) The frequency of peripheral IFN-γ- and TNF-α-producing $CD4^+$ T cells (over the gated $CD4^+$ T cell population) after *in vitro* stimulation with $ova_{(323-339)}$ (Control) or $surv_{(53-67)}$ (surv53) peptides is shown. Bars are the mean±SEM. *indicates p=0.026; ***indicates p=0.0003.

Fig S7
Lladser *et al.*

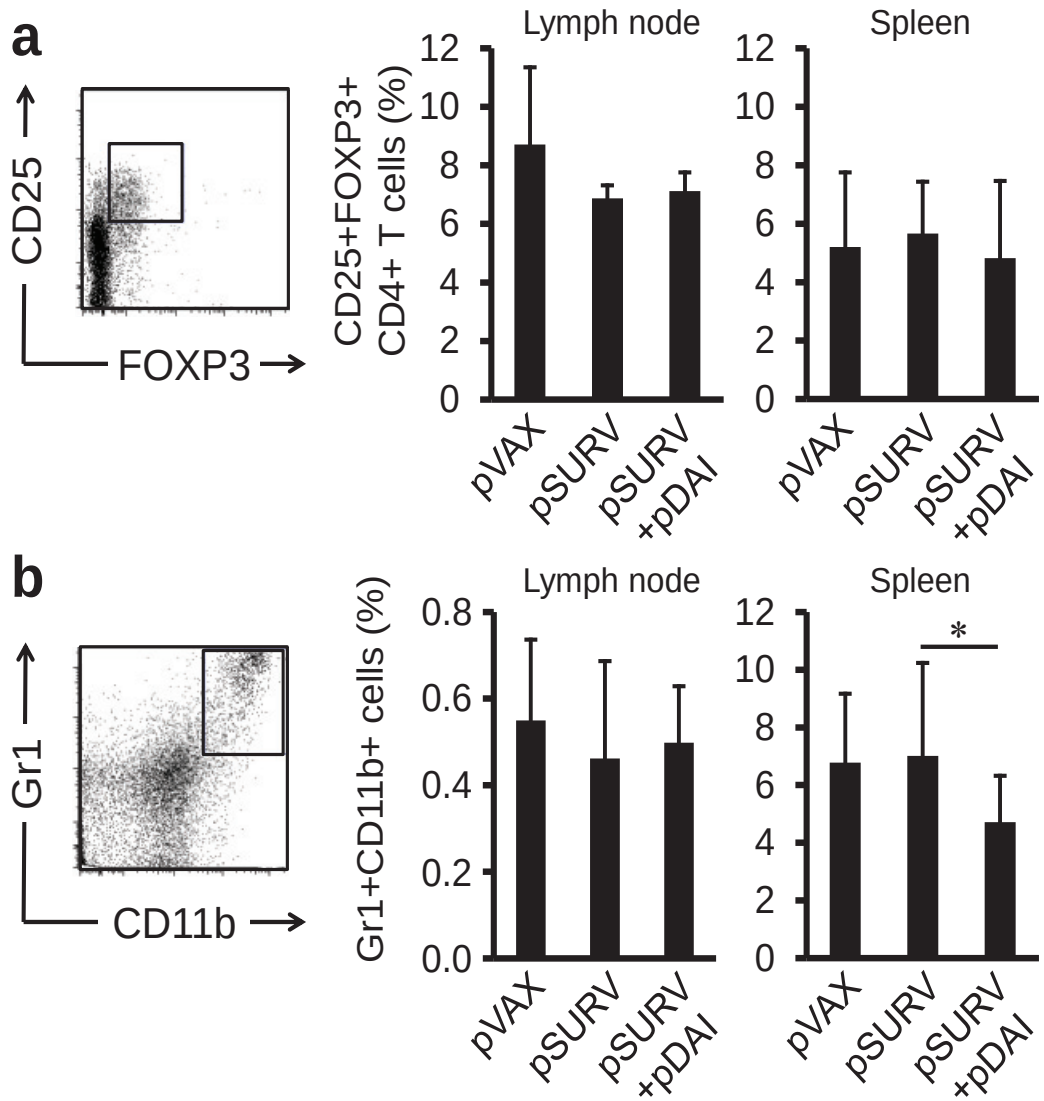

Figure S7 Analysis of immunosuppressive cell populations. C57BL/6 mice were vaccinated twice at two-week intervals with pDAI, pSURV or pSURV+pDAI (n=8). Two weeks later, T regulatory (Treg) and myeloid-derived suppressor cells (MDSC) were analyzed in spleen and inguinal lymph nodes by immunofluorescence staining and flow cytometry. The frequency of Treg ($CD4^+$; $CD25^{high}$; $FOXP3^+$) over the total $CD4^+$ T cell population (**a**) and MDSC ($CD11b^+$; $Gr1^+$) over the total cell population (**b**) were determined and the results are shown. Bars are the mean±SEM. *indicates $p<0.05$.

Table S1. The 20 most strongly upregulated gene transcripts in mice electroporated with pDAI as compared to mice electroporated with pVAX control vector.

Rank	Gene	Fold Change	Name
1	Ly75	3,48	Lymphocyte antigen 75
2	Gdf1	3,29	Growth differentiation factor 1
3	Il21	3,07	Interleukin 21
4	Ctf2	3,05	CCAAT/enhancer binding protein (C/EBP)
5	Il20	2,89	Interleukin 20
6	Ifne	2,81	Interferon epsilon
7	Il23a	2,64	Interleukin 23a
7	Il9	2,64	Interleukin 9
9	Cd40lg	2,62	CD40 ligand
10	Muc4	2,55	Mucin 4
11	Itih4	2,53	Inter-alpha inhibitor H4
12	F2	2,41	Coagulation factor II
12	Il31	2,41	Interleukin 31
14	Mpl	2,36	Myeloproliferative leukemia virus oncogene
15	Lta	2,35	Lymphotoxin alpha
16	Il13	2,33	Interleukin 13
17	Il5ra	2,31	Interleukin 5 receptor
18	Cxcr5	2,30	Chemokine (C-X-C motif) receptor 5
18	Fgf8	2,30	Fibroblast growth factor 8
20	Fgf3	2,28	Fibroblast growth factor 3
20	Il5	2,28	Interleukin 5

Table S2. Primers used for cloning and quantitative real-time PCR analysis

Primers		Sequences
Dai (cloning)	forward	5´-AGTCGAATTCCCACCATGGCAGAAGCTCCTGTTGAC-3´
	reverse	5´-AGTCGCGGCCGCTCATTGCTTGCTCAGTCCTGT-3´
Ifna (all subtypes)	forward	5´-ATGGCTAGRCTCTGTGCTTTCCT-3´
	reverse	5´-AGGGCTCTCCAGAYTTCTGCTCTG-3´
Ifnb	forward	5´-CATCAACTATAAGCAGCTCCA-3´
	reverse	5´-TTCAAGTGGAGAGCAGTTGAG-3´
Ifng	forward	5´-GCTTTAACAGCAGGCCAGAC-3´
	reverse	5´-GCAAGCACCAGGTGTCAAGT-3´
Cd40	forward	5´-CAGACACTGTGAACCCAATCAAGG-3´
	reverse	5´-TGGTGTCAGTGGCCATCTCCATAA-3´
Cd80	forward	5´-CCCCAGAAGACCCTCCTGATAG-3´
	reverse	5´-CCGAAGGTAAGGCTGTTGTTTG-3´
Ciita	forward	5´-TGCAGGCGACCAGGAGAGACA-3´
	reverse	5´-GAAGCTGGGCACCTCAAAGAT-3´
Cxcl10	forward	5´-ACTGCATCCATATCGATGAC-3´
	reverse	5´-TTCATCGTGGCAATGATCTC-3´
H2kb	forward	5´-AGGCTGGTGAAGCAGAGAGA-3´
	reverse	5´-CAGCACCTCAGGGTGACTTT-3´
Il6	forward	5´-GATGCTACCAAACTGGATATAATC-3´
	reverse	5´-GGTCCTTAGCCACTCCTTCTGTG-3´
Il10	forward	5´-GTGAAAATAAGAGCAAGGCAGTG-3´
	reverse	5´-ATTCATGGTCTTGTAGACACC-3´
Il12	forward	5´-AGTACCCTGTGCCTTGGTAG-3´
	reverse	5´-GATTCTGAACTGCGTTG-3´
Tnfa	forward	5´-CTACAGGCTTGTCACTCGAATT-3´
	reverse	5´-AATGGCCTCCCTCTCATCAGT-3´

IV

Vaccination against tumor-associated antigen Cripto-1 elicits a protective immune response to metastatic melanoma and breast cancer stem cells.

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Abstract

Cancer vaccines targeting antigens expressed by cancer stem cells (CSCs) have the potential to become potent cancer therapies. Cripto-1 (CR) is a glycoprotein that plays a critical role during embryogenesis and is over-expressed in more than 50% of human carcinomas and melanomas but is expressed only at low levels on normal differentiated tissues. CR is involved in cellular processes, which are hallmarks of CSCs and aggressive metastatic disease, such as unrestrained cell-proliferation, epithelial-mesenchymal transition and tumor-related angiogenesis. We demonstrate that a DNA vaccine-encoding mouse (m) CR is able to induce a protective immune response in mouse melanoma and breast cancer models. Vaccination of C57Bl/6 mice elicited immunological protection against lung metastasis and subcutaneous challenges with B16F10 melanoma cells. This was mediated by mCR specific CTLs for which we identified a H2-K^b restricted epitope that was effective at stimulating CD8 T cells *ex vivo* and *in vivo*. In the 4T1 metastasizing breast cancer model, vaccination against mCR reduced lung metastatic tumor burden and generated mCR specific antibodies. CR negative tumors grown in a spheroid culture system to enrich for CSCs demonstrated up-regulated CR expression and had acquired sensitivity for vaccination induced tumor rejection. In addition, vaccination with mCR in Her2 transgenic BALB-neuT mice in a therapeutic setting led to a significant reduction in metastatic spread. Our data indicate that DNA vaccination against mCR results in a protective immune response against CR expressing tumors, which could lead to the development of prophylactic and therapeutic tumor vaccines to be tested in the clinic.

Introduction

The largest hurdle in the treatment of cancer patients is not that of treating large tumors but to ensure that the disease does not disseminate in the patient, leading to metastasis and eventually death. Immunotherapy is coming to the forefront for treating metastatic disease (1) and success has been achieved with the treatment of metastatic melanoma and other tumor types using immune-checkpoint blockade (2). This releases the anti-tumor T cell immunity through removal of inhibitory signaling mediated by the PD1 or CTLA4 molecules (3). Similarly, adoptive transfer of *ex vivo* expanded and activated tumor infiltrating T cells has successfully cured a proportion of metastatic melanoma patients (4). These strategies, while potent, require the prerequisite that the patients have a pre-established anti-tumor immunity, which may frequently not be the case.

This limitation can be overcome by educating the immune system through vaccination. Vaccination has a long history in the treatment of human diseases, and while very successful in preventing pathogenic infections, less success has been achieved when applying vaccines to treat cancer. Many of the antigen targets such as MAGE-A, GP100, NY-ESO-1, Tyrosinase and Her2, have been tested in vaccination trials with limited success (5). Vaccines have been shown to elicit both humoral and cellular responses in a substantial proportion of the patients, though these responses are typically not potent enough to have an impact on the disease burden. Removing large tumor burdens through vaccination-induced antigen-specific immune responses remains a challenge.

Herein we describe that vaccination against the tumor-associated antigen Cripto-1 (CR) elicits specific immune responses and targets highly metastatic aggressive cancer models. CR is an embryonic tumor antigen and as such is related to most aspects essential for driving embryogenesis, including proliferation, angiogenesis as well as epithelial-mesenchymal transition (EMT) (6). These essential characteristics of embryogenesis resemble the aggressive traits of metastatic cancer cells and represent key hallmarks of cancer (7). Importantly, CR has been shown to be upregulated in cancer stem cell (CSC) populations in melanoma and breast cancer (8,9). Using a DNA vaccine based approach we are able to show in multiple *in vivo* models that CR can be targeted by the immune system, leading to decreased tumor burden and reduced metastatic spread. The vaccination was able to elicit CR-specific CTL responses in C57BL/6 mice, and H2-K^b restricted-epitopes were identified. In addition to a cellular response, vaccination generated an antibody response in BALB/C mice. DNA vaccines encoding mouse (m) CR (pmCR) reduced lung metastasis in an orthotopic transplanted and metastasizing breast cancer model and was capable of inhibiting dissemination of spontaneously arising breast carcinoma to the lungs in BALB-neuT mice.

Material and methods

Mice and cell lines

BALB/c, BALB-neuT and C57BL/6 mice were bred, maintained at either the Microbiology and Tumor Biology Center (Karolinska Institutet, Stockholm, Sweden) or the Molecular Biotechnology Center (University of Turin, Turin, Italy) and were handled by strict adherence to the European guidelines and University Ethical Committee. Animal studies performed in Sweden were reviewed and approved by the Regional Animal ethics committees; Stockholms Norra Djurförsoksetiska Nämnd Avdelning 2, Sweden with ethical permit number N426/11. RetV (generously donated by Prof. V. Umansky, DKFZ Heidelberg), MCA205, B16F10, B16F1, 4T1-luc, D2F2, TUBO, TSA and RMA-s were maintained with GlutaMAX-RPMI supplemented with 10% heat-inactivated FCS, 50 IU/mL penicillin, and 50 µg/mL streptomycin (Life Technologies). Cell lines were maintained at 37^{o}C with 5% CO_2 at 95% humidity and were split as was necessary using 0.05% Trypsine/EDTA (Life Technologies). To generate Cripto-1 overexpressing cell lines, mouse Cripto-1 lentiviral particles were acquired (Amsbio, Abindon, U.K.) and were used to transduce D2F2 as well 4T1-luc cells with mouse Cripto-1. Followed by cell sorting for Cripto-1 positive cells using FACs. D2F2 cells were transfected using lipofectamine 2000 (Life Technologies) with human Cripto-1 expression vector and selected using Geneticin (G418 Sulfate, Life Technologies).

Western blots

Cell lysates were prepared with 1M RIPA buffer (50nM Tris-HCl, pH 7.4, 1% Triton-X, 0.5% Na-deoxycholate, 0.1% SDS, 150mM NaCl, 2mM EDTA, 50 mM NaF) with 1x protease inhibitor (Roche, Cat. No. 04693159001) at 1x10^{6} cells/ml directly after collection from cell culture. Prior to loading on the gel protein concentrations were determined with BCA protein assay (Thermo Scientific, Rockford, IL) according to the manufacturers protocol. 20 µg protein per sample was loaded on 10% NuPAGE Bis-Tris acrylamide gels (Invitrogen) and run at 200 V for 45 minutes with MOPS SDS running buffer (Invitrogen) followed by transfer onto PVDF membrane (Immobilon-P; Millipore, Bedford, MA) for 3 hours at 40V. Blocking of the membrane was done using TBS-0.5% Tween 20 (Sigma-Aldrich), 2.5% milk powder or 2.5% BSA, followed by wash in TBS-0.5% Tween 20 and incubation with primary antibodies: rabbit α-human Cripto antibody, cross reactive to mouse 1:1,000 (Rockland, cat.no. 600-401-997) and mouse α-beta-Actin antibody 1:25,000 (Sigma-Aldrich) overnight at 4^{o}C. Secondary staining was done using α-rabbit IgG, HRP-linked (Cell Signaling Technology) and α-mouse IgG, HRP-linked (Cell Signaling Technology) for 1 hour at room temperature. Development was done using Amersham ECl Prime Western Blotting Detection Reagent (GE Healthcare). Using a LAS-1000 CCD camera system (Fujifilm, Tokyo, Japan) luminescence was detected.

Vaccination and Plasmids

Mice were treated on week 8 and week 10 either by intradermal injection of 40 µg of plasmid injected in PBS followed by electroporation protocol as described previously (10) using IGEA plate electrodes (C57BL/6, BALB/c) or by intramuscular injection of 50 µg of plasmid diluted in 20 µL of saline

followed by electroporation with IGEA array needle electrode (BALB/c, BALB-neuT) to facilitate plasmid transfer BALB-neuT mice were i.m. injected on week 10 and 12 with 50 µg plasmid and electroporated after insertion of a needle electrode in the injected area. Cripto-1 encoding plasmids were generously provided by Bianco C et. al. and cloned into the pVAX vector (Invitrogen, Carlsbad, CA, USAP) (11). Plasmid based vaccines were produced by transformation of *E. coli* (TOP10, Invitrogen) with pVAX plasmid and grown in Luria-Bertani medium containing Kanamycin (50µg/ml). To generate endotoxin-free vaccine, plasmids were purified using GigaPrep Endofree Kit (Qiagen GMBH, Hilden, Germany).

Tumor models

B16F10 tumor cells were transplanted into the C57BL/6 mice to model melanoma. B16F10 was injected s.c (50,000) or i.v. (200,000) in 100µl PBS after being harvested when *in vitro* growth was logarithmic and at 80% confluence. Tumor size was monitored by palpation with calipers and mice were sacrificed when they became moribund or when the tumor reached a volume of 1,000 mm^3. Mice that were injected i.v. were sacrificed at day 14 and lungs were excised and B16F10 foci were enumerated. 200,000 4T1-mCR cells were administered orthotopically by delivery into the mammary fat pad in 50 µl PBS. *In vivo* imaging was done with IVIS SpectrumCT (PerkinElmer) using D-Luciferin (Life Technologies), 5 µg per gram mouse was injected i.p. and allowed to disseminate in the mouse for two minutes followed by anesthesia with Isoflourane at 3% for three minutes prior to transfer onto the heated, 37^oC, SpectrumCT platform for imaging. Lungs were harvested and transferred into 4^oC PBS from the 4T1-mCR tumor bearing mice 23 days post challenge. Lungs were individually diced using scalpels in 6-well plates and dissociated in 1mg/ml DNase in StemPro Accutase Cell dissociation reagent with TrypLE at a ratio of 1:1 (Life Technologies). Diced lungs were incubated for 30 minutes in dissociation reagent and further diced with scalpels before being mechanically dissociated using a 70 µm filter (Corning). Removal of red blood cells was done using RBC lysis buffer (BioLegend) and followed by suspension in supplemented RPMI-1640 media containing 6-Thioguanine (60µM) and seeded in 150 mm cell culture dishes (Corning). After 10 days cells cell were washed with PBS, followed by formaldehyde fixation and Hematoxylin Harris (VWR, 351945S) staining for 5 minutes. Primary tumors were excised and weighed. To evaluate lung metastasis, colonies were enumerated and metastatic index was calculated. Vaccinated BALB/c mice were challenged with 10^5 TUBO cells per mouse. P3 mammospheres were cultured as described below and $2x10^4$ cells were injected s.c. into BALB/c mice. For BALB-neuT lung metastasis, lungs were harvested at approximately 28 weeks of age and fixed in paraffin followed by staining with hematoxylin and eosin. Using ImageJ metastatic index was calculated.

Immunological assays

Overlapping 15 amino acid Cripto-1 peptides were generated to cover the whole protein. RMA-s cells were washed with RPMI medium without FCS and kept at room temperature for 2 hours. $2x10^5$ cells were seeded into 96-well plates containing complete medium as well as peptides at a concentration of

100 µg/ml for 6 hours. Cells were washed and stained with α-mouse-H2-K^b-FITC (BioLegend, 116505) to detect cell surface MHC class I molecules by flow cytometry. Mouse derived peptides; mCR_{16-25} (SAFEFGPVA), mCR_{46-55} (RSFQFVPSV), mCR_{1-9} (MGYFSSSVVL), $Surv_{20-28}$ (ATFKNWPFL), $TRP2_{180-188}$ (SVYDFFVWL), $OVA_{257-264}$ (SIINFEKL) were acquired from China Peptides (ChinaPeptides Co. Ltd. Shanghai, China) at >95% purity. Peptides were used to stimulate mouse lymphocytes in peripheral blood harvested from immunized mice. Cells were seeded into 96-well plates and stimulated with 10µg/ml of MHC class I-restricted peptide. After 2 hours, GolgiPlug (Becton, Dickinson and Company) was added for the last 6 hours of stimulation. Cells were stained with α-mouse-CD8-FITC, α-mouse-IFN-γ, α-mouse-TNF-α using the Cytofix/Cytoperm Fixation/Permeabilization Solution (Becton, Dickinson and Company) according to manufacturer's instruction prior to acquisition of cells on LSRII FACS (Becton, Dickson and Company). Data were analyzed using FlowJo (Tree Star). $CD8^+$ cells were isolated from immunized mice splenocytes using MACS Beads $CD8^+$ positive selection (Miltenyi Biotec). 10^5 lymphocytes were seeded into 96-well plates and either co-cultured with $5x10^4$ B16F10 cells or peptides (10µg/ml) or non-stimulated overnight and supernatants were harvested after 18 hours. IFN-γ was evaluated using Mouse IFN-γ ELISA development kit (MabTech, Nacka Strand, Sweden) following manufactures instruction. *In vivo* cytotoxicity was evaluated as previously described (12). Splenocytes were harvested from naïve C57BL/6 mice and labeled with 0.2, lo, or 2 µM, hi, of CFSE in PBS for 5 minutes. 10% FCS containing PBS was used to halt labeling of the cells. Hi naïve target splenocytes were pulsed with target peptide (10µg/ml) and lo naïve splenocytes were pulsed with control peptide (10µg/ml). Labeled splenocytes were mixed and injected i.v. into naïve, antigen immunized (AI) or control immunized (CI) mice. After 20 hours spleens were harvested from the mice and acquired by flow cytometery. *In vivo* cytotoxicity was calculated as follows: 100 - ((percentage of hi mCR_{16-25} peptide-pulsed targets in AI or CI mice/percentage of lo control targets in AI or CI mice)/(percentage of hi mCR_{16-25} peptide-pulsed targets in naïve mice/ lo control targets in naïve mice) x 100). Antibodies from immunized mice were evaluated in serum by staining D2F2mCR cells with 1:20 dilution of serum in PBS for 20 minutes at 4^oC followed by 20 minutes of staining with α-mIgG-PE (Jackson Laboratory, 115-116-071), α-mIgG1-FITC, α-mIgG2a-FITC and α-mIgG2b-FITC (RMG1-1, RMG2a-62, RMG2b-1, BioLegend). Samples were acquired on BD LSRII and analyzed on FlowJo.

Cancer cell spheroid culture

Both TUBO and B16F10 were used in the generation of spheroid cultured tumor cells. B16F10 cells were seeded into Ultra-Low Cluster Plate (Costar) with 50,000 cells in 3 ml of melanoma spheroid culture medium. B16F10 melanoma spheroid culture medium consisted of MBM-4 (Lonza) containing the following: $CaCl_2$, bovine pituitary extract (BPE), recombinant human Fibroblast Growth Factor (rhFGF), recombinant human Insulin, hydrocortisone, PMA, GA-1000 and 10% FBS (Lonza, CC-3249). TUBO breast cancer cells were cultured in DMEM with 20% FCS prior to being transferred to ultra-low-attachment flasks (Sigma-Aldrich) at $6x10^4$ cells/ml in mammosphere medium consisting of DMEM-F12 medium (Invitrogen) with

basic fibroblast growth factor (bFGF, 20 ng/ml), recombinant human insulin (5 µg/ml), epidermal growth factor (EGF, 20 ng/ml) and bovine serum albumin (BSA, 0.4%) (Sigma-Aldrich). Cells were monitored daily and split, using enzymatic and mechanical dissociation, every third day for melanoma spheres and seventh day for mammospheres or depending on sphere aggregate cluster size. Cells were collected prior to sphere culturing and at every consecutive passage, which were denoted as P1, P2 and P3 as their passage number indicated.

Results

Vaccination with mouse Cripto-1 encoding DNA (pmCR) increases survival and reduces lung metastasis burden of B16F10 melanoma

We first analyzed the presence of CR in different mouse tumor models. Mouse tumor cell lines from C57BL/6 (B6) background were screened for mCR expression by western blot. D2F2 cell line and mCR-transfectant D2F2mCR were used as negative and positive controls for mCR expression, respectively. Significant expression was detected in B16F10 and RetV melanoma cell lines, the latter derived from the metastatic melanoma *ret* transgenic mouse model (13). B16F1, a less aggressive B16 melanoma sub-line (14,15), and the MCA205 sarcoma cell line had weaker expression of mCR (Fig. 1A, upper panel). Interestingly, when B16F10 were grown in a sphere culture system (Sup. Fig. 1A) to expand the proportion of CSCs (16,17), mCR expression was progressively increased after each round of sphere culture (Fig. 1A, lower panel). To evaluate mCR expression in healthy tissues we screened the healthy mouse tissue gene expression data set obtained by Su et. Al (GSE1133:GLP1073) (Sup. Fig. 2A) (18). As expected, within the data set we found that mCR is expressed during early embryogenesis, and expression is down regulated beyond day 8.5 into adulthood (Sup. Fig. 2B).

We next analyzed if vaccination with mCR-encoding vaccines could elicit protective immunity in B6 mice challenged with B16F10 melanoma cells. Mice were vaccinated twice with plasmid DNA delivered i.d. followed by electroporation. Two weeks after last vaccination, mice received a s.c. B16F10 tumor challenge and tumor growth was monitored. A significant delay in tumor growth was observed in pmCR-vaccinated mice, as compared to the control empty vector pVAX immunized mice (Fig. 1B). The delayed tumor growth led to significantly extended survival in pmCR-vaccinated mice (Fig. 1C). We then evaluated the ability of pmCR vaccination to protect against metastatic lung colonization of i.v. injected B16F10 cells. A significant decrease in the number of metastatic lung colonies (Fig. 1D) and total tumor foci (Fig. 1E) was observed in mice vaccinated with mCR. These results indicate that vaccines encoding CR induce immunity with the potential to target highly metastatic melanoma cells.

Generation of tumor antigen-specific $CD8^+$ responses in pmCR immunized mice

To establish whether mCR-specific T cell immune responses were generated by the vaccination, we first defined the tumor epitopes recognized by the resulting $CD8^+$ T cells. A library consisting of 33 long overlapping peptides (15-mers) derived from the mCR amino acid sequence was screened for the ability to bind MHC class I molecules H2-K^b using the RMA-s MHC class I stabilization assay. Three of the peptides tested were able to stabilize H2-K^b at the cell surface of RMA-S cells (mCR_{1-15}, mCR_{16-30} and mCR_{46-60}, Fig. 2A), and as expected, the positive control H2-K^b epitopes, $Trp2_{180-188}$ (19) and $OVA_{257-264}$, strongly stabilized H2-K^b. We then identified the presence of MHC

class I-restricted epitopes within these 15-mer peptides using an *in silico* prediction analysis (http://www.cbs.dtu.dk/services/NetMHCpan/) (Sup. Table 1). The predicted 9-mer epitopes were then tested for their ability to be recognized by peripheral blood $CD8^+$ T cells in vaccinated mice. Indeed, two of these predicted peptides, mCR_{16-25} (Fig. 2B) and mCR_{46-55}, elicited an *ex vivo* IFN-γ and TNF-α response after peptide stimulation. No responses specific for mCR_{1-9} were detected (Sup. Fig 3). The serum from vaccinated B6 mice contained no detectable antibodies specific for mCR (data not shown). We further confirmed the ability of $CD8^+$ T cells isolated from spleens of vaccinated animals to secrete significant amounts of IFN-γ in response to mCR_{16-25} peptide and tumor cell stimulation but not after control stimulation with OVA peptide or left unstimulated (Fig. 2C). The ability of mCR_{16-25}-specific $CD8^+$ T cells to mediate cytotoxic killing was further evaluated *in vivo* by transferring target spleen cells pulsed with either mCR_{16-25} or OVA control peptide and stained with high or low concentrations of CFSE, respectively. Specific killing of the mCR_{16-25} pulsed $CFSE^{hi}$ relative to the internal control of OVA pulsed $CFSE^{lo}$ splenocytes was analyzed by flow cytometry one day after transfer to mice. Killing of mCR_{16-25} pulsed cells was observed to be significantly greater in mCR vaccinated mice than in control mice (45% ± 1% versus 2% ± 1%) (Fig. 2D, E). These results demonstrated that mCR_{16-25} specific CTLs were able to elicit *in vivo* cytotoxic killing.

Vaccination against mCR increases survival and reduces lung metastasis burden in mammary carcinoma models.

To extend our study also to mammary carcinomas, four mouse mammary carcinoma cell lines on BALB/c background were screened by western blot for expression of mCR. In contrast to the melanoma cell lines none of the mammary carcinoma lines expressed high amounts of mCripto-1, though weak bands could be identified for 4T1 (20), TS/A and TUBO (21), while the D2F2 (22) line was negative for mCR expression. mCR transfectants were generated by transfecting the mCR negative D2F2 cell line with vectors encoding for mCR. These transfectants were used as positive controls for mCR expression. As a first approach to establish the protective potential of mCR vaccination-induced immune responses over the dissemination of mammary cancer cells in BALB/c models, we generated a stable mCR 4T1-luc transfectant (4T1-mCR) (Fig. 3A), which was used as a model for spontaneous lung metastasis. BALB/c mice were vaccinated twice with pmCR or control pVAX plasmids prior to implantation of 4T1-mCR cells into the mammary fat pad. Primary tumor growth was evaluated by *in vivo* luciferase activity detection at day 14 (Fig. 3B) and by measuring tumor weight (Fig. 3C) at day 23 after tumor challenge. In mice vaccinated with pmCR tumor growth was significantly reduced as compared to pVAX mice. Furthermore, pmCR vaccination greatly reduced spontaneous metastasis to the lungs as evaluated by a colony formation assay (Fig. 3D). The humoral response was evaluated as the ability of serum antibodies to bind mCR on D2F2mCR transfectants; mCR negative D2F2 cell line were used as negative control. We were able to identify mCR specific IgG antibodies in pmCR-vaccinated mice while not in pVAX control mice (Fig. 4A). We further evaluated the subclasses of mCR specific antibodies that were generated as measured by flow

cytometry analysis using anti-IgG1, IgG2a and IgG2b – FITC conjugated antibodies (Fig. 4B). The predominant subclass detected in the serum was IgG2.

To further validate pmCR vaccination strategy in a breast cancer setting relying on the endogenous expression of the antigen, TUBO cells, typically negative or low for mCR expression (Fig. 5A), were cultured in a sphere culture system using low attachment flasks to enrich for the small population of tumor cells with enhanced metastatic potential. These CSCs have been shown to be difficult to treat with classical therapies and (23) have been identified to express increased levels of CR (8,24,25). The mCR expression was found to progressively increase upon continuous passages under sphere forming conditions (Fig. 5A). The third passage (P3) of TUBO derived mammospheres exhibited higher mCR expression than either P1 or P2 (Fig. 5A). We next tested the potential of pmCR vaccination against both TUBO cells grown in normal conditions and TUBO P3 spheres. As anticipated, vaccination with pmCR had no beneficial effect against mCR negative TUBO tumors. No increased survival (Sup. Fig. 4) or change in tumor growth was observed (data not shown). In contrast, pmCR vaccination induced decreased tumor growth (Fig. 5B) and prolonged survival (Fig. 5C) in mice challenged with TUBO derived P3 cells, as compared to the control vaccinated group.

We finally tested the therapeutic potential of pmCR vaccination in a more clinically relevant mouse model. BALB-neuT female mice (26), which develop mammary carcinoma with a stepwise progression that mimics several features of human *ErbB2* carcinogenesis (27) with the appearance of lung metastases from the 6^{th} month onwards (28). Mice were vaccinated twice with pmCR or control plasmid at 10 and 12 weeks of age. In this setting, the immune response generated by the vaccine was not able to control the development of primary tumors, and consequently no differences in overall survival were observed between the groups (Fig. 6A). In addition to primary tumors, lungs were also harvested to evaluate the spontaneous metastatic spread of the developing tumors (Fig. 6B). Lungs were stained with hematoxylin and eosin to enumerate metastases and the metastatic index was calculated. Interestingly, pmCR vaccinated BALB-neuT mice displayed a significant suppression of the metastatic burden as compared to control vaccinated mice (Fig 6C). These results led us to conclude that pmCR vaccination efficiently induces antigen-specific immune responses able to target metastatic spreading in different preclinical models of melanoma and breast cancer.

Discussion

Our current understanding of the complex nature of tumors has recently established that a small population of cancer cells within the heterogeneous tumor mass is particularly efficient in initiating the formation of disseminated cancerous lesions. These cells are referred to as cancer initiating cells or CSCs and have been shown to be difficult to eliminate with classical therapies(23). The development of therapies selectively targeting these CSCs to prevent metastatic disease therefore is of considerable importance. We demonstrate here that CR, which has been described to be upregulated in human breast CSC (24), can be used to target CSCs in the setting of a cancer vaccines.

Prophylactic vaccination has been a success story in protecting us from a plethora of pathogens, but unfortunately the success seen in this context has not been translated well to therapeutic vaccination against cancer. Perhaps it should not be expected that vaccine-induced anti-tumor immune responses would be capable of removing bulky tumors, particularly when they consist of a heterogeneous cancer cell population that mediate a strong immunosuppression (29). Instead, vaccines should enable the immune system to reach distant sites of disease that evade the capability of traditional therapies. It is therefore essential that anti-cancer vaccines focus on the elimination of metastatic CSC populations. This study shows the potential of targeting the tumor-associated antigen CR with a plasmid DNA based vaccination approach capable of eliciting an immune response that in particular can inhibit aggressive tumor growth related to metastatic spread and cancer CSC initiated tumorigenesis.

CR has been shown to be expressed on many different tumors (6), including uveal and cutaneous melanomas as well as breast cancer (30-32). Expression of CR indicates a poor prognosis for breast cancer patients (33) and CR positive tumor cells are endowed with an aggressive tumorigenic phenotype (34) which affects many of the pathways expected to be up-regulated in metastatic malignant melanoma. Although CR has yet to be described for human metastatic melanoma, we identified several mouse B6 metastatic melanoma cell lines positive for mCR. In mice that were vaccinated with i.d. delivered and electroporated DNA plasmids encoding mCR we found that growth of the highly aggressive B16F10 cell line was significantly inhibited. Of particular interest, prophylactic vaccination against mCR led to a marked reduction in the metastatic spread of B16F10 tumor cells to lungs. While immunization against CR leads to inhibition of both i.v. and s.c. injection of B16F10, these routes of delivery of tumor cells do not adequately model the different stages of EMT that tumors undergo (35). As an oncofetal antigen, CR through its interactions with TGF-beta ligands, nodal and glypican-1, initiates the transformation of cells to a state prepared for EMT (6). In MMTV-CR transgenic mice, mammary lesions demonstrated loss of E-cadherin and gain of N-cadherin as well as vimentin leading to EMT (36). To model mammary carcinoma derived metastasis we employed the 4T1 orthotopic mouse model. After screening 4T1 as well as other mouse breast cancer cell lines, we found them all to be low- or none- expressers of mCR. This may be

explained by the down-regulated expression of CR found on long-term *in vitro* cultured breast cancer cell lines (24). This led us to produce stably transformed mCR expressing 4T1 cells, which we subsequently injected into the mammary fat pad post vaccination and monitored tumor development. While there was a moderately significant impact on luminescence, as measured two weeks after challenge, there was a very significant reduction of the metastatic index in the pmCR vaccinated mice compared to control vaccinated mice.

CR is expressed on CSC populations from human metastatic melanoma cells (8), prostate cancer cells (25) and breast cancer cells (24). The promoter region of CR has been found to contain binding sites for the CSC markers Oct-4 and Nanog (37). Previously we (C. L., S.L., and F.C.) have cultured breast carcinoma cells, derived from the BALB-neuT mouse, in mammospheres and have found an up regulation of Oct-4, among many other CSC markers (9). Here we confirmed an increase in CR expression as a CSC marker upon passaging of the BALB-neuT mouse tumor TUBO on low adherent plates. A graded number of passage three TUBO mammospheres were transplanted s.c. into pmCR vaccinated and control vaccinated mice. pmCR vaccinated mice were able to control the growth of the tumors while control vaccinated were not, increasing their overall survival significantly.

To confirm the therapeutic potential of CR-specific immunity elicited by DNA based vaccination, a more clinically relevant mouse model was used. We vaccinated BALB-neuT mice, which spontaneously develop tumors in the mammary tissue driven by rat Neu, a Her2 ortholog. At four weeks of age these mice display atypical hyperplasia in the mammary ducts that develop into *in situ* carcinomas by week eight (38). In this setting we therapeutically vaccinated the mice at 10 weeks of age. This led to significantly reduced metastatic burden in the lungs but not to increased overall survival (Fig. 6). Reducing metastatic burden represents the potential that vaccination against tumor-associated antigens has when unleashed on a small population of metastasizing cells.

DNA vaccines have the potential to become a vaccine delivery method for a plethora of different diseases, but as yet much is to be desired from the clinical results generated by DNA based trials (39). The key to the potential of DNA vaccines lies in their ability to elicit both a cellular as well as a humor response to antigens encoded by the plasmids. Cytotoxic T lymphocytes are essential in an effective anti-tumor immune response (40). Herein, we are the first to describe a cytotoxic T cell response to mCR (Fig. 2). CR, as well as being a GPI anchored extracellular protein, interacts with Notch1 in the ER/golgi apparatus (41). This lends itself to MHC class 1 immunosome processing. By identifying strong stabilizing partners for H2-K^b with the help of the TAP-1 deficient RMA-s cell line (42) we were able to identify potential CTL epitopes and confirm them *in silico*. Splenocytes from vaccinated mice were able to generate IFN-γ and TNF-α responses to two out of the three predicted epitopes. We further validated that mCR_{16-25} is an epitope that $CD8^+$ T cells could react to by isolating specific $CD8^+$ T cells from the vaccinated mice, and further confirmed that also epitope specific killing *in vivo* is effective. In B6

mice we were able to raise T cells specific for mCR, but were unsuccessful in raising a humoral response (data not shown). In BALB/c mice the opposite was true, with a predominant humoral response with a dominant isotype of IgG2a and IgG2b. This suggests that antibody dependent cellular cytotoxicity (ADCC) may play a role in the protection generated by vaccination in mice of this background. Antibodies to CR have previously been shown to be effective in mouse models of cancer therapy. Adkins *et. al* were able to show that CR antibodies were able to block Nodal and Act-B interaction, leading to reduced tumor growth (43). Antibodies specific to CR were also able to induce cell death in doxorubicin resistant leukaemias (44). While we suspect that ADCC may play a role in the antibody response generated by our DNA vaccine, *Kelly et. al* could target CR expressing tumors with an antibody cytotoxic conjugate in a setting where no functional immune system is required (45). This antibody has undergone clinical trials (NCT00674947) but no results have been published yet (as of March 2015).

In summary, our findings show that targeting CR using DNA vaccination elicits beneficial humoral and cellular immunity capable of significantly reducing aggressive metastatic as well as CSC based tumor growth. We identify, for the first time, CTL epitopes specific to mCR, and have in ongoing work also several human CTL epitopes defined. The possibility of targeting CR expressed on the small CSC population is attractive, particularly in an adjuvant setting to avoid metastatic spread.

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Figure 1. Vaccination against mCR increases survival and reduces metastatic lung burden in B6 mice challenged with mCR expressing B16F10. A, upper panel: tumor cell lysates from lines on B6 background, RetV, MCA205, B16F10 and B16F1 were checked by western blot for mCR expression and compared relatively to beta-actin. The D2F2 mCR transfectant (D2F2mCR) was included as positive control. A, lower panel, B16F10 tumor cells were grown on low attachment plates for one to three passages (P1, P2 and P3) to enrich for tumor initiating cells and cell lysates were generated from each passage. B, survival of B6 mice (n=10 per group) prophylactically vaccinated with pmCR or control vector pVAX with two doses of 20 µg plasmid DNA delivered i.d. with electroporation prior to s.c. challenge with $5x10^4$ B16F10 cells, compared with Mantel-Cox test; **, $P < 0.01$. The results shown here are representative of two experiments. C, tumor growth was compared in mice vaccinated with either pmCR or pVAX with 2-way ANOVA test; **, $P < 0.01$. D, representative photos of B16F10 metastatic lung burden in vaccinated mice harvested 14 days post i.v. challenge of $2x10^5$ tumor cells. E, reduction in lung metastasis was evaluated by enumerating foci of tumors in lungs of B16F10 i.v. challenged mice and compared with non-parametric T test; *, $P < 0.05$. The results shown here are representative of three experiments.

Figure 2. Vaccination with pmCR elicits an epitope specific functional $CD8^+$ T cell response. A, RMA-s cells were used to measure surface stabilization of H2-K^b using an overlapping 15-mer peptides library. H2-K^b was analyzed by flow cytometery after loading with 100 µg/ml of peptide for 2 hours, with negative controls (un-stained, filled gray, and un-load, green), positive controls (OVA and TRP2, red) as well as the peptides mCR_{46-60}, mCR_{16-30}, mCR_{1-15} (solid black). C, peripheral blood lymphocytes from vaccinated mice (n=9) were stimulated with mCR_{16-25} specifically for 8 hours prior to ICS and significant increase in IFN-γ/TNF-α producing cells were found; ***, $P < 0.001$ using Mann-Whitney T test. D, $CD8^+$ T cells were isolated using MACs bead positive selection from B6 (n=5) mice vaccinated with either pmCR or pVAX and stimulated with CR expressing tumor cell line B16F10, mCR_{16-25} peptide, OVA peptide or with T cells alone; **, $P < 0.01$ using Mann-Whitney T test. E, splenocytes from naïve mice were harvested and labeled with lo and hi CFSE prior to loading with OVA and mCR_{16-25}, respectively, and transferred into pmCR or pVAX vaccinated mice. Specific killing was analyzed by FACS after 20 hours. F, mCR_{16-25} specific killing of splenocytes *in vivo* was found to be significantly greater in pmCR vaccinated mice; ***, $P < 0.001$ using Students T test.

Figure 3. In the orthotropic 4T1-mCR breast cancer model metastatic spread is inhibited by prophylactic pmCR vaccination. A, Tumor cell lines from BALB/c background were screened for mCR expression using western blot. Stable mCR transfectants expressing luciferase were generated from the 4T1 and D2F2 breast carcinoma cell lines. B, 4T1-mCR was injected orthotopically in the mammary fat pad in BALB/c mice that had been vaccinated two weeks before with either pmCR or pVAX. *In vivo* luminescence was measured 14 days post orthotopic 4T1-mCR challenge of vaccinated pmCR and pVAX (n=12) mice, a Mann-Whitney T test was used; **, $P < 0.01$ Mann-Whitney T

test. C, tumors were excised on day 23 after tumor challenge and weight was compared between pVAX and pmCR vaccinated mice; ***, $P < 0.001$ Mann-Whitney T test D, spontaneous metastasis derived from orthotopically transplanted tumors were evaluated by colony formation assay and the weight of the primary tumor in vaccinated mice, a Mann-Whitney T test was used; ***, $P < 0.001$. The results shown here are representative of two experiments.

Figure 4. Vaccination of BALB/c mice induces the production of mCR specific antibodies. A, BALB/c mice were vaccinated, as described in material and methods, and serum was harvested 14 day after the final vaccination. Serum from mice was then used to stain control CR negative cells (D2F2) and mCR positive D2F2mCR at a dilution of 1:20. Cells were then stained with secondary anti-mouse IgG-PE antibody followed by acquisition by flow cytometry. B, similarly, D2F2mCR were stained with pooled serum (1:20) derived from pmCR vaccinated mice and stained with either anti-mouse IgG1, anti-mouse IgG2a or anti-mouse IgG2b. Total MFI and the relative contribution of each antibody to this staining were calculated.

Figure 5. pmCR vaccination protects against breast CSC challenge but not against parental cell line. A, TUBO cells were passaged on low attachment plates to generate P1, P2 and P3 mammospheres. Cell lysates were generated from these cultures and screened for mCR expression by western blot and relative mCR expression was compared by beta-actin control. B, $2x10^4$ P3 mammospheres derived from TUBO parental cells were injected s.c. into pmCR and pVAX vaccinated mice and tumor growth was compared. On day 69 evaluated tumor size was significantly different as calculated by Mann-Whitney T test; *, $P < 0.05$. C, Survival was monitored in BALB/c mice challenged with P3 mammospheres post vaccination, compared with Mantel-Cox test; *, $P < 0.05$.

Figure 6. Vaccination of BALB-neuT mice against mCR shows protection against the spread of Her2/*neu* driven spontaneous metastasis to the lungs. A, BALB-neuT mice (n=6 per group) were vaccinated at 10 weeks of age, followed by a boost at 12 weeks; mice survival was monitored and compared between pmCR and pVAX vaccinated mice. B, representative sections of lungs harvested from BALB-neuT mice and stained for tumors with hematoxylin and eosin when ethical endpoint had been reached. C, metastatic index were enumerated and compared between pmCR vaccinated and pVAX mice, a Mann-Whitney T test was used; *, $P < 0.05$.

Fig 1

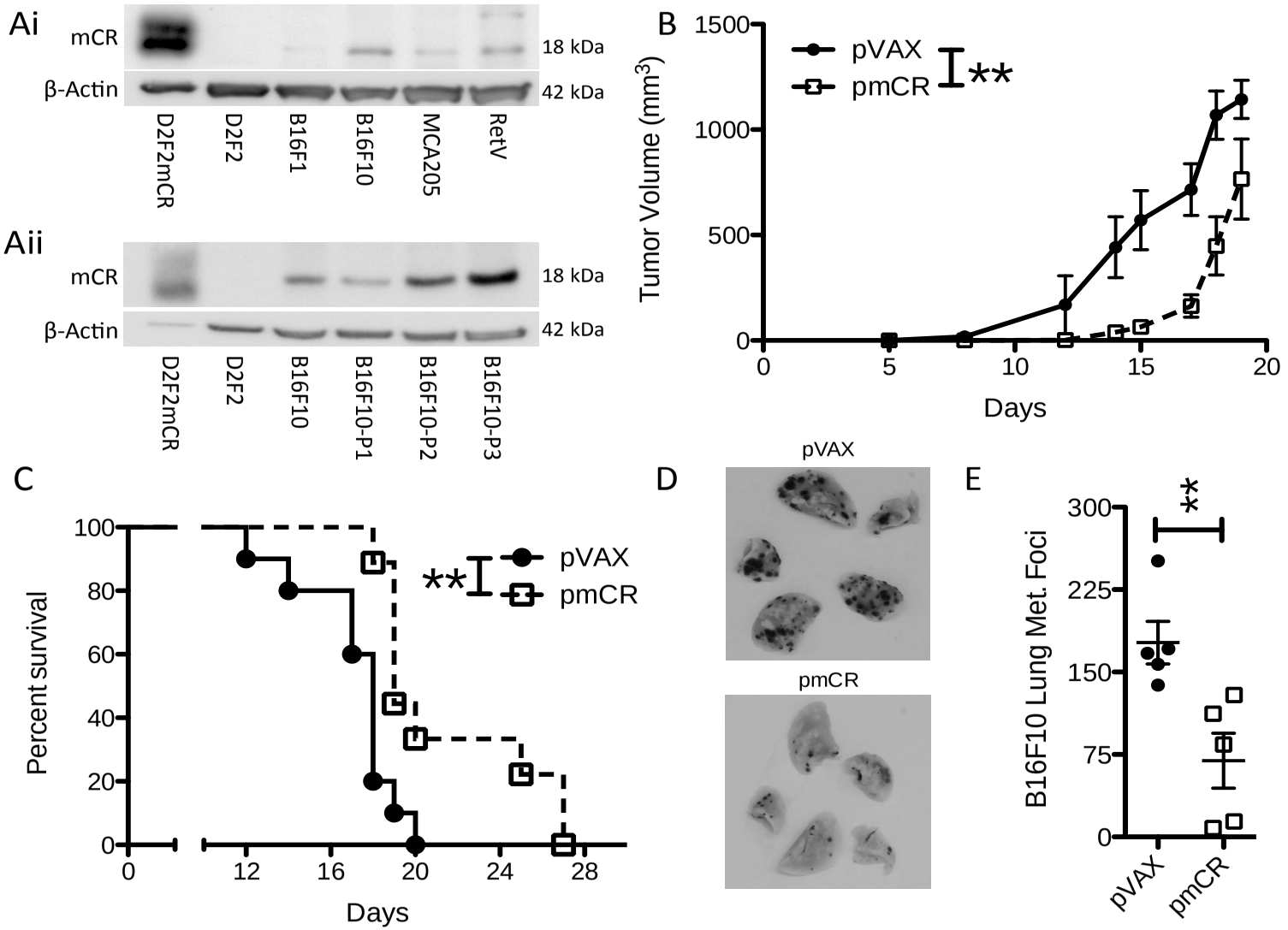
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Fig 2

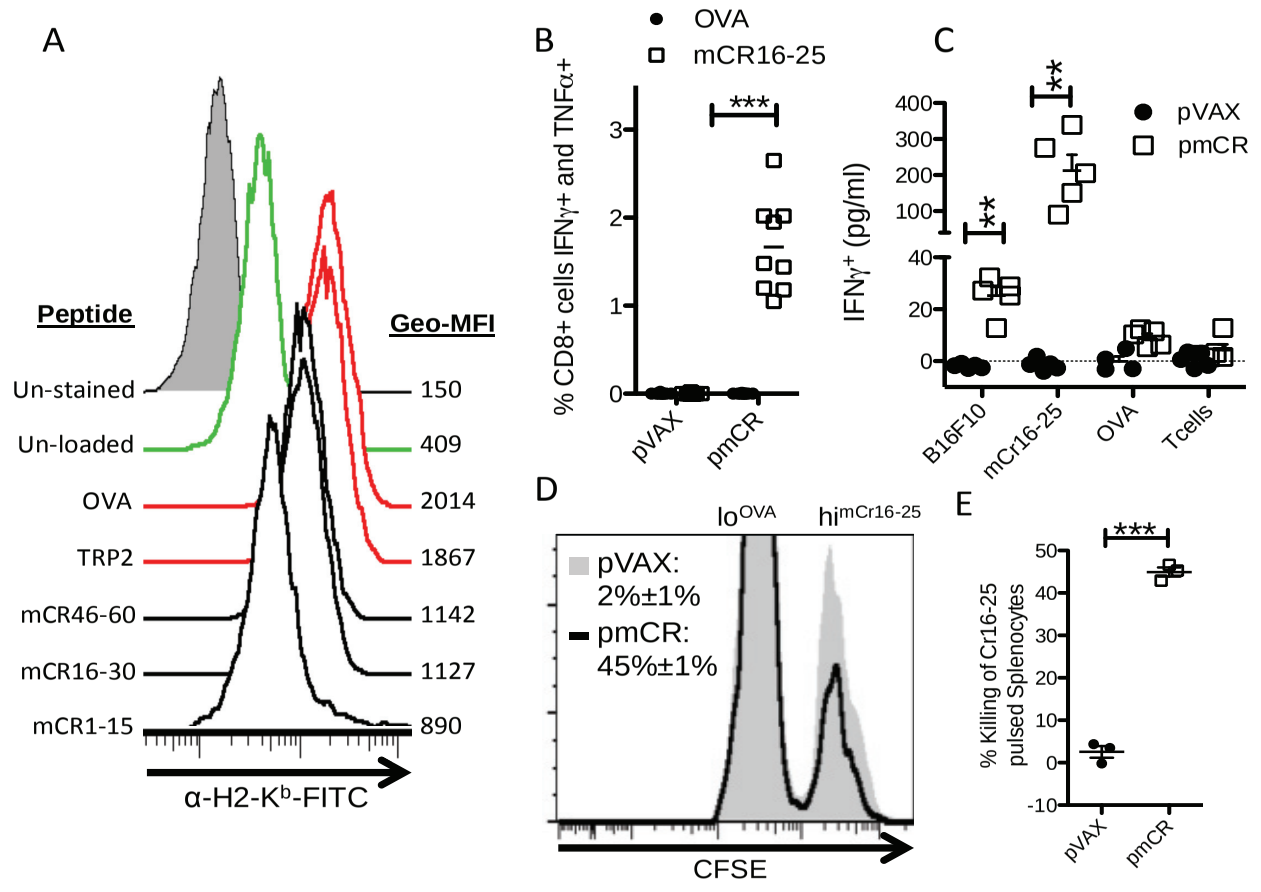
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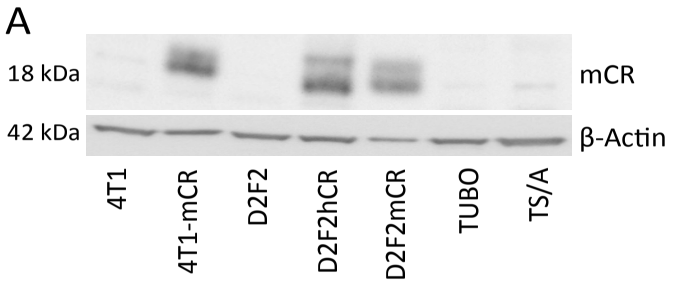
Fig 3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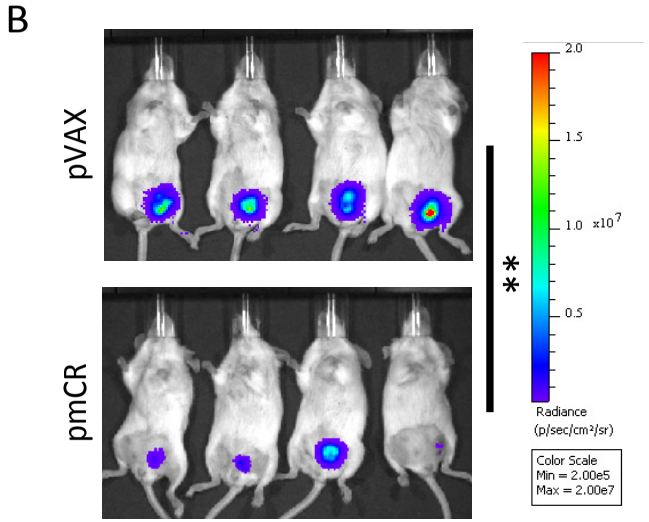
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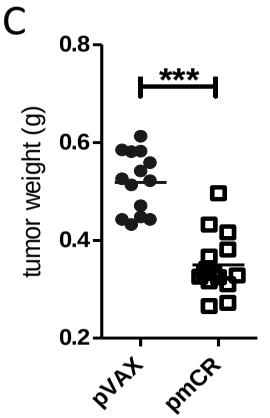
Min = 2.00e5
Max = 2.00e7


C
tumor weight (g)
0.8
0.6
0.4
0.2
***
pVAX
pmCR

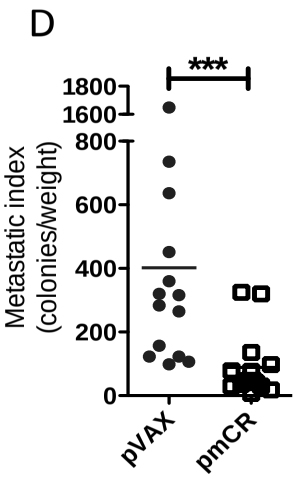

D
Metastatic index
(colonies/weight)
1800
1600
800
600
400
200
0
***
pVAX
pmCR

Fig 4

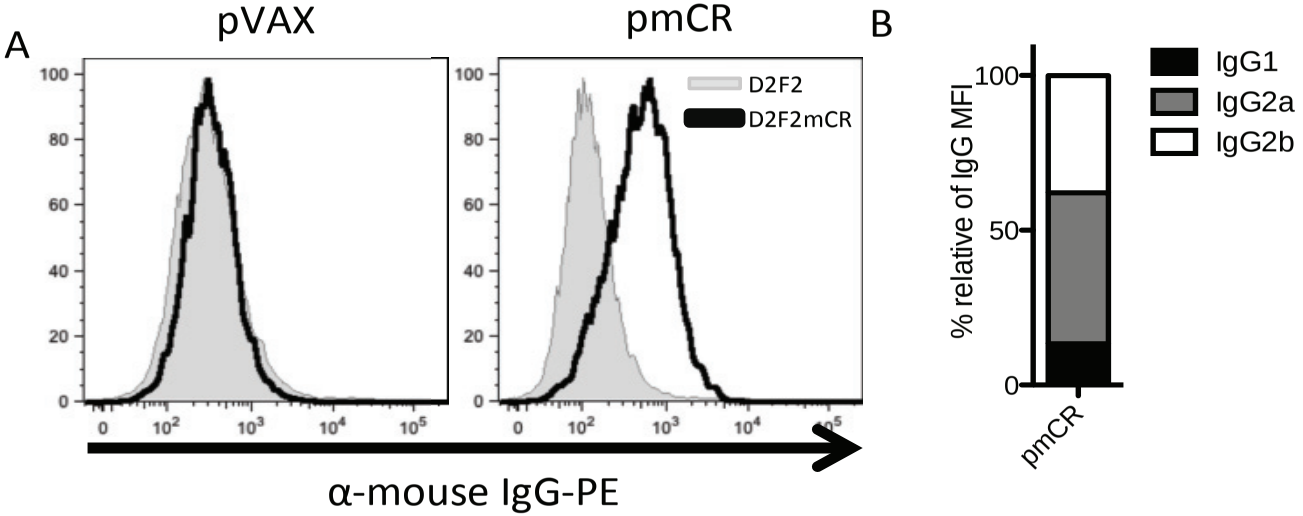
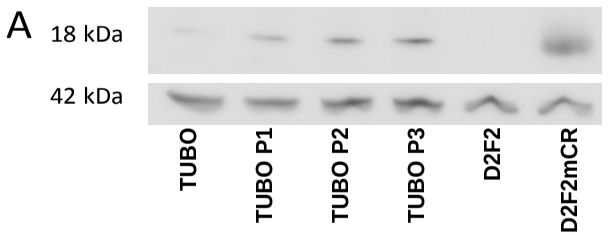
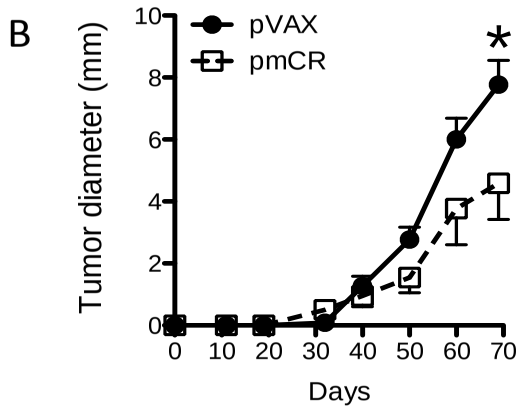

A
pVAX
pmCR
D2F2
D2F2mCR
100
80
60
40
20
0
0
10^2
10^3
10^4
10^5
α-mouse IgG-PE
B
% relative of IgG MFI
100
50
0
pmCR
IgG1
IgG2a
IgG2b

Fig 5


A
18 kDa
42 kDa
TUBO
TUBO P1
TUBO P2
TUBO P3
D2F2
D2F2mCR


B
Tumor diameter (mm)
pVAX
pmCR
*
0
2
4
6
8
10
0 10 20 30 40 50 60 70
Days

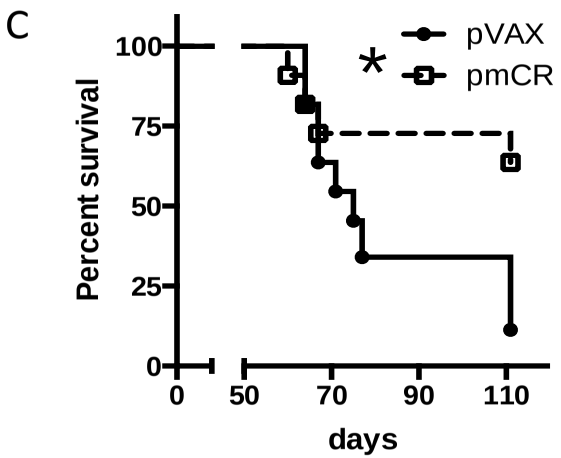
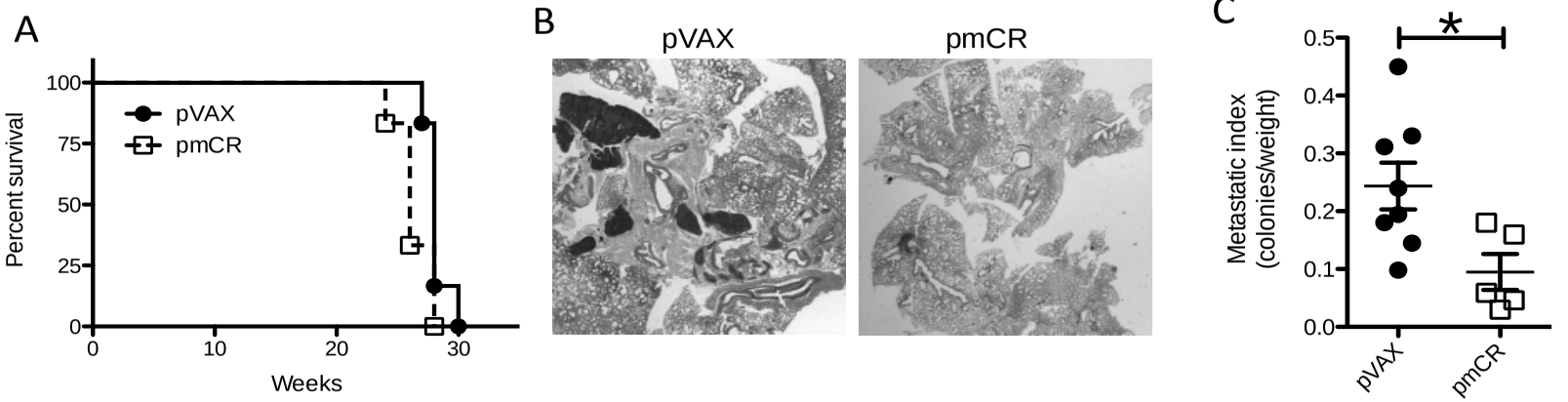
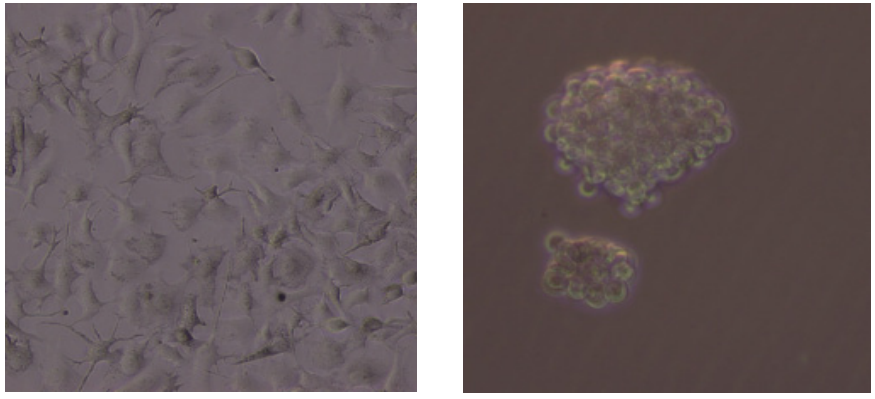

C
Percent survival
pVAX
pmCR
*
0
25
50
75
100
0 50 70 90 110
days

Fig 6


A
100
75
50
25
0
Percent survival
pVAX
pmCR
0
10
20
30
Weeks
B
pVAX
pmCR
C
0.5
0.4
0.3
0.2
0.1
0.0
Metastatic index
(colonies/weight)
*
pVAX
pmCR

Sup. Fig 1

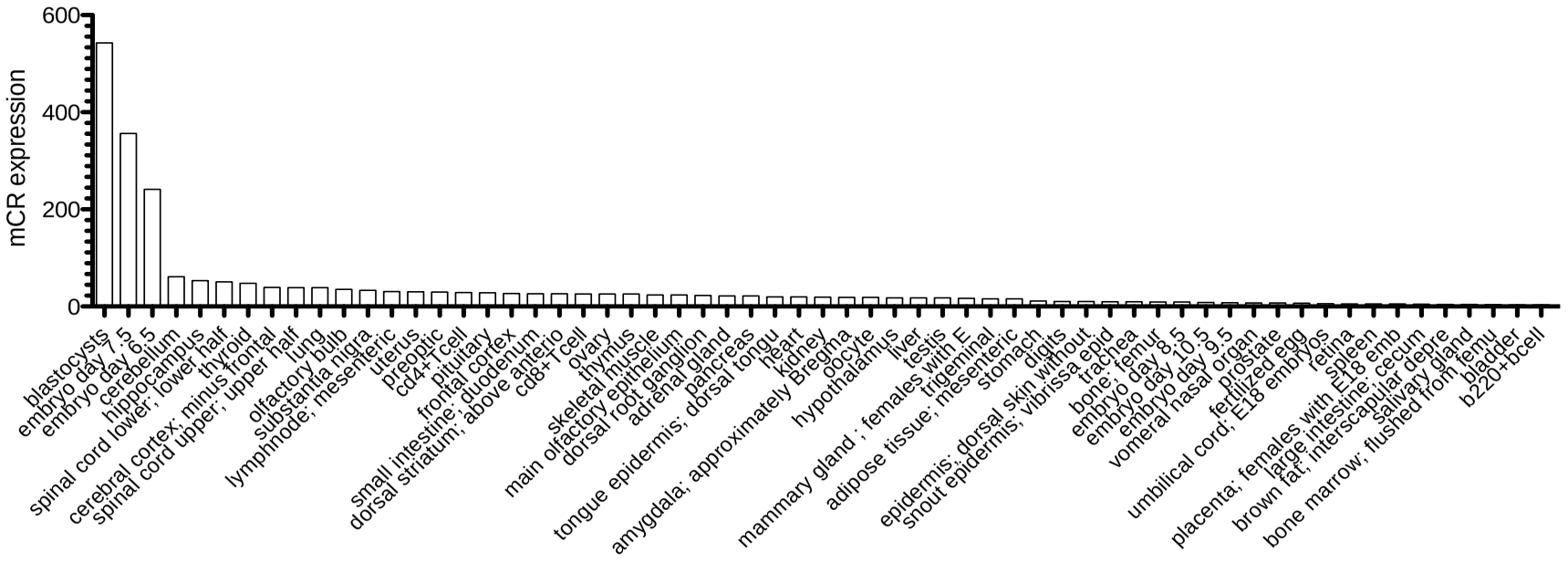


B16F10 B16F10 P1

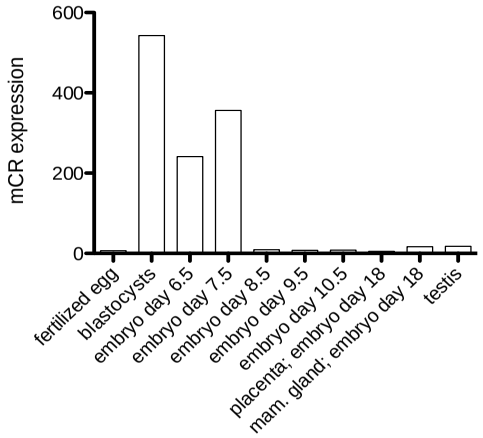
Suppl. Figure 1. Light microscopy of adherent B16F10 monolayer culture and B16F10 spheres culture. A, Light microscopy image of adherent B16F10 monolayer culture, 20x magnification, after 72h culture. B, Light microscopy image of P1 B16F10 spheres, 20x magnification, after 48h culture on low attachment plates.

Sup. Fig 2

A



B

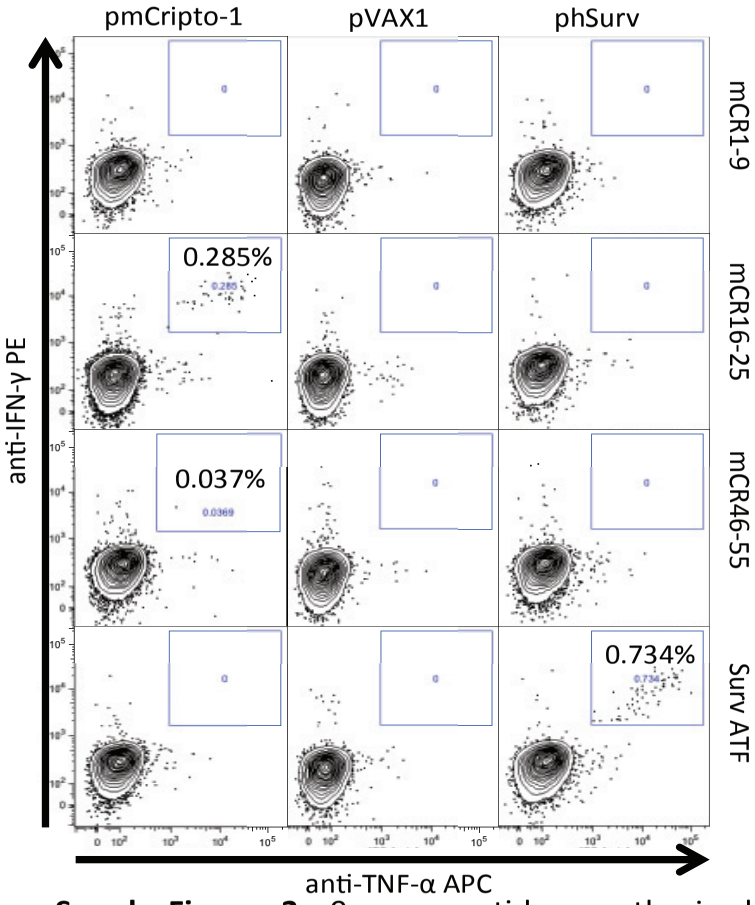


Suppl. Figure 2. Screening for Cripto-1 in healthy tissue mRNA. A, Expression levels of mCR were assessed from healthy mouse tissue from the GSE1133 data set and represented sorted with highest expression to the left. B, Expression profile of mCR during embryogenesis

Sup. Table 1

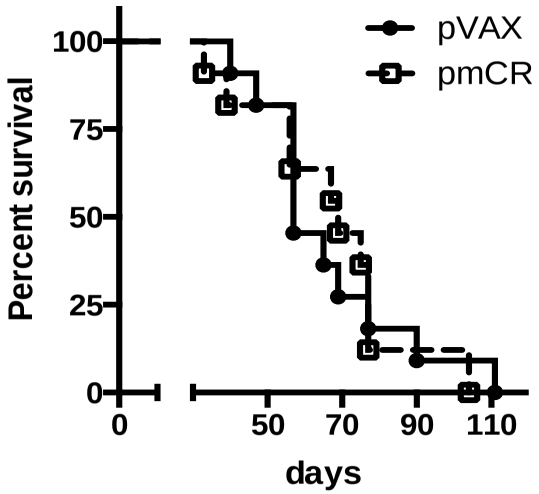
Peptide	nM	Rank	Strong/Weak
mCR46-55	84,8772	0,25	SB
mCR16-25	345,4973	1,5	WB
mCR1-9	560,4001	2	WB

Sup. Fig 3



Suppl. Figure 3. 9-mer peptides synthesized based on RMA-s stabilization and NetMHC *in silico* H2-K^{b} prediction were used to stimulate peripheral blood lymphocytes from pmCR, pVAX and phSurv vaccinated mice for 8 hours prior to intracellular staining for IFN-γ and TNF-α in CD8^{+} T cells.

Sup. Fig 4



Suppl. Figure 4. BALB/c mice vaccinated with either pmCR or pVAX were challenged with 10^5 TUBO cells s.c. and survival was monitored over 110 days.

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